Definition of the sigma(W) Regulon of Bacillus subtilis in the Absence of Stress

Zweers, Jessica C.; Nicolas, Pierre; Wiegert, Thomas; van Dijl, Jan Maarten; Denham, Emma L.

Published in:
PLoS ONE

DOI:
10.1371/journal.pone.0048471

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Zweers, J. C., Nicolas, P., Wiegert, T., van Dijl, J. M., & Denham, E. L. (2012). Definition of the sigma(W) Regulon of Bacillus subtilis in the Absence of Stress. PLoS ONE, 7(11), [e48471].
https://doi.org/10.1371/journal.pone.0048471

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Absence of Stress

PLOS ONE | www.plosone.org 1 November 2012 | Volume 7 | Issue 11 | e48471

Department of Medical Microbiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands, 1
INRA, UR1077, Mathématique Informatique et Génome, Jouy-en-Josas, France, 2 Hochschule Zittau/Görlitz, FN/Biotechnologie, Zittau, Germany

Jessica C. Zweers1, Pierre Nicolas2, Thomas Wiegert3, Jan Maarten van Dijl1,4, Emma L. Denham1

Abstract

Bacteria employ extracytoplasmic function (ECF) sigma factors for their responses to environmental stresses. Despite intensive research, the molecular dissection of ECF sigma factor regulons has remained a major challenge due to overlaps in the ECF sigma factor-regulated genes and the stimuli that activate the different ECF sigma factors. Here we have employed tiling arrays to single out the ECF σW regulon of the Gram-positive bacterium Bacillus subtilis from the overlapping ECF σX, σY, and σM regulons. For this purpose, we profiled the transcriptome of a B. subtilis sigW mutant under non-stress conditions to select candidate genes that are strictly σW-regulated. Under these conditions, σW exhibits a basal level of activity. Subsequently, we verified the σW-dependency of candidate genes by comparing their transcript profiles to transcriptome data obtained with the parental B. subtilis strain 168 grown under 104 different conditions, including relevant stress conditions, such as salt shock. In addition, we investigated the transcriptomes of rasP or prsW mutant strains that lack the proteases involved in the degradation of the σW anti-sigma factor RsiW and subsequent activation of the σW-regulon. Taken together, our studies identify 89 genes as being strictly σW-regulated, including several genes for non-coding RNAs. The effects of rasP or prsW mutations on the expression of σW-dependent genes were relatively mild, which implies that σW-dependent transcription under non-stress conditions is not strictly related to RasP and PrsW. Lastly, we show that the pleiotropic phenotype of rasP mutant cells, which have defects in competence development, protein secretion and membrane protein production, is not mirrored in the transcript profile of these cells. This implies that RasP is not only important for transcriptional regulation via σW, but that this membrane protease also exerts other important post-transcriptional regulatory functions.

Citation: Zweers JC, Nicolas P, Wiegert T, van Dijl JM, Denham EL (2012) Definition of the σW Regulon of Bacillus subtilis in the Absence of Stress. PLoS ONE 7(11): e48471. doi:10.1371/journal.pone.0048471

Editor: Adam Driks, Loyola University Medical Center, United States of America

Received July 6, 2012; Accepted September 26, 2012; Published November 14, 2012

Copyright: © 2012 Zweers et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: JZC, PN, JMvD, and ELD were in parts supported by the European Science Foundation under the EUROCORES Programme EuroSCOPE, grant 04-Escope 01-011 from the Research Council for Earth and Life Sciences of the Netherlands Organization for Scientific Research, the CEU projects LSHM-CT-2006-019064, LSHG-CT-2006-037469, PITT-GA-2008-215524 and 244093, and by the transnational SysMO initiative through projects BACELL SysMO1 and 2. TW was supported by the Deutsche Forschungsgemeinschaft (WI 1771/5-1). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: j.m.van.dijlj01@umcg.nl

Introduction

Extracytoplasmic function (ECF) sigma factors enable bacteria to respond adequately to harsh and stressful environmental conditions. The numbers of ECF sigma factors vary among different bacteria. While some bacteria (e.g. Mycoplasma genitalium) have no ECF sigma factors, other bacteria can contain over 50 (Streptomyces coelicolor). In most cases however, only a limited number of ECF sigma factors are present. For example, Escherichia coli produces 2, and Bacillus subtilis 7 [23]. In non-stressed cells, these sigma factors are usually inhibited by binding to a specific anti-sigma factor [49]. For several anti-sigma factors it has been shown that specific extracellular stresses trigger their regulated intramembrane proteolysis (RIP) by site-1 and site-2 proteases in the membrane [22,24,29,44]. Specifically, the site-1 protease clips in the extracytoplasmic part of the anti-sigma factor and renders it a substrate for the intramembrane cleaving site-2 protease. This results in the release of the anti-sigma factor/sigma factor complex into the cytoplasm, where the anti-sigma factor is further degraded and the sigma factor can then redirect transcription [10,21,23,27,29]. Attempts to accurately define each of the ECF sigma factor regulons in organisms with multiple ECF sigma factors have been complicated by partial overlaps that exist both for the binding sites recognized by these sigma factors and the stimuli that activate them. This is very clearly illustrated by studies on the σW, σX, σY and σM sigma factors and their regulons in B. subtilis [8,9,14,25,26,33,34,45]. To single out the individual ECF sigma factor regulons is challenging, which is underscored by a recent classification of the promoters of B. subtilis based on an unsupervised algorithm [35]. This approach, which involved transcript profiling across 104 different conditions, only allowed the identification of a global ECF regulon, while the individual σW, σX, σY and σM regulons remained undefined.

The σW regulon is among the three best-studied ECF sigma factor regulons in B. subtilis. This regulon is induced in response to cell envelope stress caused by antibiotics, alkaline shock and salt shock [8,9,18,31,38,39,43,48]. The anti-sigma factor of σW, RsiW, is cleaved by the site-1 protease PrsW and the site-2 protease RasP [12,15,21,42,49]. Consistent with the requirement of PrsW for RsiW degradation, prsW mutant cells have a phenotype that is very similar to the phenotype of sigW mutant cells. In contrast,
deletion of the rasP gene causes a pleiotropic phenotype including defects in the development of competence for genetic transformation and protein secretion [20,41]. Although transcriptional analyses with sigW mutant cells were previously performed [8], a detailed comparison of the effects of a sigW mutation with those of prsW or rasP mutations on genome-wide transcription has not yet been documented. Additionally, in the previous transcriptional analyses of the sigW deletion strain, non-coding RNAs (ncRNAs) were not included. Therefore, the present studies were aimed at defining the strictly σW-regulated genes by transcript profiling studies with tiling arrays using RNA from sigW, prsW or rasP mutant strains. Notably, these array analyses were performed in the absence of stress stimuli because, under these conditions mutant strains. Notably, these array analyses were performed in the absence of stress stimuli because, under these conditions σW-regulated genes expressed at very low level might be missed. The results thus obtained were enriched using data from the B. subtilis transcript profiling study with tiling arrays in which gene expression in the parental strain 168 was assessed under 104 different biological conditions [35].

Materials and Methods

Bacterial strains, plasmds and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown in Luria Bertani (LB) medium (Difco Laboratories) at 37°C with vigorous shaking. Overnight grown pre-cultures in LB medium were diluted to an OD_{600} of 0.05 in fresh LB medium and then grown to the exponential phase as determined by optical density readings. Under these conditions σW is active but the cells are not stressed.

RNA isolation

Samples for three biological replicates of each mutant and the parental strain 168 were produced by independent culturing, harvesting of the bacterial cells, and RNA isolation. When the cultures reached an OD_{600} of 1.0 the equivalent of 15 OD units of cells were harvested and total RNA was isolated according to Eymann et al., 2002 [16] with some minor modifications. Cell culture samples were added to 0.5 volume of frozen killing buffer (20 mM Tris-HCl [pH 7.5], 5 mM MgCl2, 20 mM NaN3) and centrifuged for 10 min at 4°C. The cell pellets thus obtained were frozen in liquid nitrogen and stored at −80°C. Pellets were resuspended in 200 μl ice-cold killing buffer and transferred to precooled Teflon disruption vessels filled with liquid nitrogen. Cells were then disrupted for 2 min at 2600 rpm in a Mikro-Dismembrator S (Sartorius). The frozen powder was resuspended in 4 mL prewarmed (50°C) lysis solution (4 M guanidine thiocyanate, 25 mM sodium acetate [pH 5.2], 0.5% N-laurylsarcosinate [wt/vol]) and immediately frozen in liquid nitrogen. Total RNA was isolated by acid-phenol extraction. Samples were extracted twice with 1 volume of acid phenol/chloroform/isoamyl alcohol (25:24:1, [pH 4.5]) and once with 1 volume of chloroform/isoamyl alcohol (24:1). After adding 1/10 volume of 3 M sodium acetate (pH 5.2), RNA was precipitated overnight with isopropanol at −20°C. Precipitated RNA was washed with 70% ethanol and dissolved in 100 μl of RNase free water. The isolated RNA was DNase-treated using the RNase-Free DNase Set (Qiagen) and purified using the RNA Clean-Up and Concentration Micro Kit (Norgen). RNA concentrations were measured using a Nanodrop-1000 spectrophotometer and RNA quality was assessed with the Agilent 2100 Bioanalyzer according to the manufacturer’s instructions. Labeling of the samples and hybridizations were performed in strand-specific conditions by NimbleGen, as previously described [40], using Basysbio_T2 tiling arrays (NimbleGen). All tiling array data can be queried under the NCBI-GEO accession numbers GSE35236 and GPL15150.

Statistical analyses

An aggregated expression measure was computed for each annotated and for each transcribed segment recently identified in the systematic study of transcriptome changes across lifestyles [35]. This measure consists of the median of the smoothed signal for probes with a unique perfect match on the genome sequence lying entirely within the boundaries of a particular feature [35]. The data was quantile-normalized to remove trends caused by technical variations between experiments [5]. A single linear model was fitted on the log2-scale data to assess the links between variations of expression and the genetic background of the analyzed sigW, rasP or prsW mutant strains and the parental strain 168. The p-values associated with the tests for non-null effects of each mutation compared to the parental strain were computed (function “lm” in R). One of the three hybridizations for the prsW mutant harbored an atypical transcript profile resembling that of RNA extracted from stationary phase cells. We interpret this observation as the result of a technical error when the samples were prepared, and this data point was therefore discarded. From the p-values, q-values allowing the control of the false discovery rate were estimated using the procedure of Strimmer [3] as implemented in the R package “fdrtool”. To increase the statistical power of our analyses, we also considered computation of false discovery rates using the same procedure, but restricting our attention to the subset of genes that were previously predicted as part of the global ECF regulon [35].

Expression profiles across 104 conditions and ECF sigma factor binding site predictions

In addition to our transcript profiling experiments with mutant strains, we used the data from a study on the B. subtilis 168 transcriptome across 104 biological conditions (269 hybridizations), that was aimed at covering the maximum diversity of this bacterium’s lifestyles [35]. These included growth on various media and carbon-sources, responses to stresses and developmental processes, such as competence development and the sporulation-germination cycle. In particular, we incorporated in our analysis the newly identified transcription segments, such as antisense RNAs and putative regulatory ncRNAs. For a high-level comparison of expression profiles, we relied on a classification based on average-linkage hierarchical clustering of the matrix of pairwise correlation with a cut-off set to 0.4 that defined 167 high-level clusters numbered in an arbitrary order C1 to C167. To complement the list of genes previously reported as being controlled by an ECF sigma factor, we also used the results of an un-supervised classification of the sequences upstream tran-

Table 1. B. subtilis strains.

| Strain | Genotype | Reference |
|--------|----------|-----------|
| 168    | trpC2    | (35)      |
| sigW   | trpC2 sigW::bleo, Bm’ | (42) |
| rasP   | trpC2 rasP::tet, Tet’ | (42) |
| prsW   | trpC2 prsW::bleo, Bm’ | (21) |

doi:10.1371/journal.pone.0048471.t001

PLOS ONE | www.plosone.org 2 November 2012 | Volume 7 | Issue 11 | e48471
scription start sites that identified 79 putative ECF sigma-factor dependent promoters [35].

**Results**

Two groups of down-regulated genes in \(\text{sigW}\) mutant cells

Several previously documented studies have employed different strategies to identify genes that are regulated by \(\sigma^W\) [2,6,8,9,25,26,34,46]. To accurately define the \(\sigma^W\) regulon and to include possible ncRNAs that are controlled by \(\sigma^W\) under non-stress conditions, we analyzed the genes that are down-regulated in the \(\text{sigW}\) mutant compared to the parental strain with tiling arrays (GEO accession number GSE35236). To ensure that genes not related to \(\sigma^W\) activity were excluded from this study, we made use of the fact that \(\sigma^W\) becomes active in the late exponential growth phase under non-stress conditions [25]. This is important because the absence of a stress stimulus provides a unique opportunity to obtain an untroubled view of the \(\sigma^W\) regulon since stress-related side effects on the entire regulatory network are largely absent. As expected, most genes previously designated as part of the \(\sigma^W\)-regulon were down-regulated in our tiling array analysis of the \(\text{sigW}\) mutant compared to the parental strain. However, we observed that the effect amplitudes varied considerably between these genes, which allowed us to distinguish three subgroups (Figure 1, Tables 2 and 3). Group 1 consists of genes that are strongly down-regulated (this group has effect values ranging in log2-scale from \(-4\) to \(-1.5\)). The most strongly down-regulated genes belonging to group 1 are \(\text{rsiW}\) and \(\text{spo0M}\). Group 2 contains previously reported \(\sigma^W\)-regulated genes that are less strongly down-regulated due to the \(\text{sigW}\) mutation than the genes in group 1 (effect-values between \(-1.5\) and \(-0.2\)). Group 3 consists of 16 genes that were previously reported as \(\sigma^W\)-regulated, but that nonetheless were not down-regulated in the present transcriptome analyses of the \(\text{sigW}\) mutant. Based on the present data, we identified 89 potentially \(\sigma^W\)-regulated genes, which are located in 28 operons (Tables 2 and 3). The division of genes into groups 1 and 2 did not correlate with the transcription levels of these genes in the parental strain (Mann-Whitney U-test p-value = 0.23). This rules out the possibility that the observed bimodal pattern of down-regulation of genes in the \(\text{sigW}\) mutant is simply a reflection of their transcription levels in the parental strain. Indeed, the apparently bimodal down-regulation pattern of gene expression in the \(\text{sigW}\) mutant probably results from more complex transcriptional regulation. Of the 28 identified \(\sigma^W\)-regulated operons, 12 consist only of group 1 genes, and 8 consist only of group 2 genes. In 8 operons a combination of group 1 and group 2 genes was found, the group 2 genes always being localized at the end of these operons. In many cases, the boundary between group 1 and 2 genes correlated with the presence of an internal promoter (before \(\text{yozO}\), \(\text{yhlP}\), \(\text{S161}\), \(\text{ysxH}\), \(\text{yofO}\), \(\text{S659}\), \(\text{S716}\)), or a terminator (after \(\text{yhlO}\), \(\text{yvdD}\), \(\text{yxvE}\), \(\text{yqfB}\)) that could potentially be responsible for differences in their responses to the \(\text{sigW}\) deletion [35]. We also examined the sequences corresponding to predicted ECF sigma factor binding sites [35] upstream of the genes of group 2 to those of group 1, but could not identify differences in the sequences that would explain the observed behavior.

Genes that were found to be statistically significantly down-regulated in the \(\text{sigW}\) mutant are likely to be regulated by \(\sigma^W\). To establish this list of genes we computed q-values from the p-values, which allowed us to control the number of false positive identifications by taking into account the high number of genes examined. Based on this statistical analysis, we propose that genes down-regulated in the \(\text{sigW}\) mutant with q-values lower than 0.05 are most likely genuine \(\sigma^W\)-regulated genes (Table 2; genes with q-values<0.05 are marked with *). However, if we consider only these genes as being \(\sigma^W\)-regulated, several genes that were previously shown to be \(\sigma^W\)-regulated by other methods (Table S1) would have to be discarded from the \(\sigma^W\) regulon under non-stress conditions despite their apparent down-regulation. To avoid such

![Figure 1. Effect values for transcriptional changes in sigW mutant B. subtilis cells.](Image)

The transcript abundance in \(\text{sigW}\) mutant cells was compared to that in the parental strain 168 by tiling array analyses. The effect values were calculated on a log2 scale and the numbers of genes with a particular effect value were plotted as a function of the effect values. The black line represents all analyzed genes. The dashed line represents only the genes that are statistically significantly downregulated in the \(\text{sigW}\) mutant. The grey line represents the genes that were previously reported as being \(\sigma^W\)-regulated. The groups 1, 2 and 3 of \(\sigma^W\)-regulated genes are indicated.

doi:10.1371/journal.pone.0048471.g001
Table 2. Down-regulated genes in sigW mutant cells.

| Name               | Effect sigW/WT | Function                                  | Regulators                     | Genetic organization | Cluster | $\sigma^{\text{WYX}}$ promoter sequence | Conclusion |
|--------------------|----------------|-------------------------------------------|--------------------------------|----------------------|---------|------------------------------------------|------------|
| rsiW               | −0.68*         | Control of sigW activity $\sigma^{\text{Y}}$, AbrB | sigW-rsiW                      | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| sigW               | −0.63*         | Sigma W factor $\sigma^{\text{Y}}$, AbrB | sigW-rsiW                      | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| spo0M              | −0.42*         | Sporulation $\sigma^{\text{Y}}$, $\sigma^{\text{Z}}$ | spo0M                          | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| S691               | −3.66*         | S691-yoaG-S690                            |                                | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| yeaA               | −3.61*         | $\sigma^{\text{Y}}$, $\sigma^{\text{Z}}$, DegU | yeaA-yeaP-yeaQ               | C2                   | Yes     | Secondary $\sigma^{\text{W}}$           |            |
| ysdB               | −3.54*         | $\sigma^{\text{Y}}$, $\sigma^{\text{Z}}$ | ysdB                           | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| yjoB               | −3.40*         | $\sigma^{\text{Y}}$                       | yjoB                           | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| ydjP               | −3.34*         | $\sigma^{\text{Y}}$, $\sigma^{\text{Z}}$ | yeaA-yeaP-yeaQ               | C2                   | Yes     | Secondary $\sigma^{\text{W}}$           |            |
| S692 (indep)       | −3.23*         |                                         |                                | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| yxlJ               | −3.12*         | $\sigma^{\text{Y}}$, $\sigma^{\text{Z}}$, DegU | S1495-ylxJ-ylxK                | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| yoaG               | −3.07*         | $\sigma^{\text{Y}}$                       | S691-yoaG-S690                  | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| fosB               | −3.03*         | Fosfomycin resistance $\sigma^{\text{Y}}$ | fosB-S658-S659                  | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| ythP               | −2.98*         | ABC transporter (ATP binding protein) $\sigma^{\text{Y}}$ | ythP-ythQ                      | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| S690               | −2.90*         | S691-yoaG-S690                            |                                | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| S1495 (indep)      | −2.89*         | S1495-ylxJ-ylxK                          |                                | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| ythQ               | −2.74*         | ABC transporter $\sigma^{\text{Y}}$        | ythP-ythQ                      | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| S742               | −2.70*         |                                         | S742-yeaA-S740-S739-yocM       | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| pspA               | −2.68*         | $\sigma^{\text{Y}}$, AbrB | pspA-yeaD-yeaE-yeaF             | C6                   | Yes     | Secondary $\sigma^{\text{W}}$           |            |
| yflH               | −2.52*         | SdpC resistance $\sigma^{\text{Y}}$, $\sigma^{\text{Z}}$ | yflH-yflM                     | C5                   | Yes     | Secondary $\sigma^{\text{W}}$           |            |
| ydgG               | −2.51*         | $\sigma^{\text{Y}}$, AbrB | pspA-yeaD-yeaE-yeaF             | C6                   | Yes     | Secondary $\sigma^{\text{W}}$           |            |
| S719 (inter)       | −2.49*         |                                         | yobI-S719                      | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| S658 (inter)       | −2.48*         |                                         | yobI-S658-S659                  | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| yshB               | −2.47*         | $\sigma^{\text{Y}}$, AbrB | yshB-yshP-yshQ                  | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| ydbT               | −2.47*         | $\sigma^{\text{Y}}$                       | ydbT-S567-S562-secA-secB-secC | C6                   | Yes     | Secondary $\sigma^{\text{W}}$           |            |
| ydbS               | −2.46*         | $\sigma^{\text{Y}}$                       | ydbT-S567-S562-secA-secB-secC | C6                   | Yes     | Secondary $\sigma^{\text{W}}$           |            |
| pspE               | −2.33*         | $\sigma^{\text{Y}}$                       | pspE-secA                      | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| yoaG (floT)        | −2.33*         | Sporulation (early stage) $\sigma^{\text{Y}}$ | yoaG-yoaH-yoaI                 | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| yfhM               | −2.30*         | Survival of ethanol stress $\sigma^{\text{Y}}$, $\sigma^{\text{Z}}$ | yfhM-yfhM                     | C5                   | Yes     | Secondary $\sigma^{\text{W}}$           |            |
| ydhT               | −2.27*         | $\sigma^{\text{Y}}$, AbrB | pspA-yeaD-yeaE-yeaF             | C6                   | Yes     | Secondary $\sigma^{\text{W}}$           |            |
| yflB               | −2.25*         | Resistance against sublucin $\sigma^{\text{Y}}$ | yoeZ-yoeA-yoeB-yeaC-yeaD       | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| yobJ               | −2.24*         | $\sigma^{\text{Y}}$                       | yobJ-S719                      | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| yoeZ               | −2.21*         | Serine protease, Resistance against sublucin $\sigma^{\text{Y}}$ | yoeZ-yoeA-yoeB-yeaC-yeaD       | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| ydjI               | −2.17*         | $\sigma^{\text{Y}}$, AbrB | pspA-yeaD-yeaE-yeaF             | C6                   | Yes     | Secondary $\sigma^{\text{W}}$           |            |
| racX               | −2.12*         | Control of biofilm formation $\sigma^{\text{Y}}$ | racX                           | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| yoaF               | −2.11*         | Resistance against sublucin $\sigma^{\text{Y}}$ | yoeZ-yoeA-yoeB-yeaC-yeaD       | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| mtlF               | −2.05*         | Uptake of mannitol MtTR | mtlF-mtlI-mtlD                  | C36                  | No      | background                                |            |
| yuaI               | −2.02*         | $\sigma^{\text{Y}}$                       | yuaI-yuaG-yuaI                  | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| mtlD               | −1.97*         | Mannitol utilization MtTR | mtlF-mtlI-mtlD                  | C36                  | No      | background                                |            |
| ywiA               | −1.91*         | $\sigma^{\text{Y}}$, AbrB | ywiA-ywiB-ywiC-ywiD-S1338       | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| ywB                | −1.85*         | $\sigma^{\text{Y}}$, AbrB | ywiA-ywiB-ywiC-ywiD-S1338       | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| mtlA               | −1.85*         | Mannitol utilization MtTR | mtlF-mtlI-mtlD                  | C36                  | No      | background                                |            |
| ywIE               | −1.82*         | $\sigma^{\text{Y}}$                       | ywIE-S1390                      | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| yuaF               | −1.78*         | $\sigma^{\text{Y}}$                       | yuaI-yuaG-yuaI                  | C9                   | Yes     | core $\sigma^{\text{W}}$                |            |
| yoaF               | −1.58*         | $\sigma^{\text{Y}}$                       | yoaI-yuaG-yuaI                  | C48                  | Yes     | Secondary $\sigma^{\text{W}}$           |            |
Table 2. Cont.

| Name             | Effect sigW/WT | Function | Regulators | Genetic organization | Cluster | σMMW promoter sequence | Conclusion |
|------------------|----------------|----------|------------|----------------------|---------|------------------------|------------|
| $S160$ (inter)   | $-1.56$        |          |            |                      | C6      | Yes                    | Secondary $σ^W$ |
| ybpP             | $-1.38^*$      |          | $σ^B$, AcrB |                      | C9      | Yes                    | core $σ^W$   |
| $S89^*$          | $-1.34^*$      |          |            |                      | C9      | Yes                    | core $σ^W$   |
| yviD             | $-1.34$        |          | $σ^{99}$AcrB |                      | C9      | Yes                    | core $σ^W$   |
| yviC             | $-1.32$        |          | $σ^{10}$AcrB |                      | C9      | Yes                    | core $σ^W$   |
| yijH             | $-1.19$        |          |            |                      | C9      | Yes                    | core $σ^W$   |
| sppA             | $-1.18$        |          | $σ^W$      | sppA-yteJ             | C6      | Yes                    | Secondary $σ^W$ |
| yteJ             | $-1.17^*$      |          | $σ^B$      |                      | C6      | Yes                    | Secondary $σ^W$ |
| yaaN             | $-1.11$        |          | $σ^W$      | xpaC-yaaN-S22         | C9      | Yes                    | core $σ^W$   |
| yceE             | $-1.04$        | Resistance against | $σ^W$, $σ^B$, $σ^M$ | $S106$-yceC-yceD-yceE-yceF-yceG-yceH | C6 | Yes | Secondary $σ^W$ |
| $S716$           | $-0.95$        |          |            |                      | C31     | Yes                    | Read through |
| $S659$ (indep)   | $-0.94$        |          |            | lfoS-S658-S659        | C9      | Yes                    | core $σ^W$   |
| yceD             | $-0.90$        | Resistance against | $σ^W$, $σ^B$, $σ^M$ | $S106$-yceC-yceD-yceE-yceF-yceG-yceH | C6 | Yes | Secondary $σ^W$ |
| yceH             | $-0.88^*$      |          | $σ^W$, $σ^B$, $σ^M$ | $S106$-yceC-yceD-yceE-yceF-yceG-yceH | C6 | Yes | Secondary $σ^W$ |
| $S22$ (intra)    | $-0.88$        |          |            |                      | C9      | Yes                    | core $σ^W$   |
| yceG             | $-0.87^*$      |          | $σ^W$, $σ^B$, $σ^M$ | $S106$-yceC-yceD-yceE-yceF-yceG-yceH | C6 | Yes | Secondary $σ^W$ |
| yceC             | $-0.84$        |          | $σ^W$, $σ^B$, $σ^M$ | $S106$-yceC-yceD-yceE-yceF-yceG-yceH | C6 | Yes | Secondary $σ^W$ |
| yxeA             | $-0.83$        |          | S-box      | Downstream of S1495-xysJ-yxjF | C48 | Yes | Read through |
| ypsA             | $-0.82$        |          | Opposite of spo0M | No | | Background |
| $S1338$          | $-0.80$        |          |            | lfoS-yviF-yviC-yviD-S1338 | C9 | Yes | core $σ^W$   |
| $ivD$            | $-0.78$        | Aminoacid biosynthesis | CodY | No background | | |
| yknX             | $-0.78$        | Resistance against SdpC | $σ^{99}$AcrB | yknW-yknX-yknY-yknZ | C9 | Yes | core $σ^W$   |
| $S106$           | $-0.78$        |          |            | $S106$-yceC-yceD-yceE-yceF-yceG-yceH | C6 | Yes | Secondary $σ^W$ |
| xpaC             | $-0.77$        |          | $σ^W$      | xpaC-yaaN-S22         | C9      | Yes                    | core $σ^W$   |
| yafC             | $-0.76^*$      |          | $σ^B$      | yafE-yafA-yafB-yafC-yafD | C2 | Yes | Secondary $σ^W$ |
| yknY             | $-0.76$        | Resistance against SdpC | $σ^{99}$AcrB | yknW-yknX-yknY-yknZ | C9 | Yes | core $σ^W$   |
| $S1175$          | $-0.75$        |          | S-box      | S’ mntA               | C1      | No | background |
| ycfF             | $-0.74$        |          | $σ^{99}$, $σ^B$, $σ^M$ | $S106$-yceC-yceD-yceE-yceF-yceG-yceH | C6 | Yes | Secondary $σ^W$ |
| yqfD             | $-0.72^*$      |          | $σ^B$      | yafE-yafA-yafB-yafC-yafD | C2 | Yes | Secondary $σ^W$ |
| yknZ             | $-0.69$        | Resistance against SdpC | $σ^{99}$AcrB | yknW-yknX-yknY-yknZ | C9 | Yes | core $σ^W$   |
| mtnK             | $-0.65$        |          | S-box      | mtnK-mtnA             | C48 | No | background |
| alsD             | $-0.63$        |          |            | alsS-alsD             | C39 | No | background |
| yoaO             | $-0.60$        |          | $σ^{99}$   | $S742$-yoaO-S740-S739-yocM | C9 | Yes | core $σ^W$   |
| yknW             | $-0.57$        | Resistance against SdpC | $σ^{99}$AcrB | yknW-yknX-yknY-yknZ | C9 | Yes | core $σ^W$   |
| $S740$ (inter)   | $-0.54$        |          |            | $S742$-yoaO-S740-S739-yocM | C6 | Yes | Secondary $σ^W$ |
| $S61$            | $-0.52$        | Fatty acid biosynthesis | $σ^B$ | acpS | C3 | Yes | Secondary $σ^W$ |
| $S739$           | $-0.51$        |          |            | $S742$-yoaO-S740-S739-yocM | C2 | Yes | Secondary $σ^W$ |
| $S1390$ (inter)  | $-0.48$        |          |            | yvrF-S1390            | C9 | Yes | core $σ^W$   |
| $S442$ (inter)   | $-0.48$        |          |            | yjeH-S442             | C9 | Yes | core $σ^W$   |
| acpS             | $-0.45$        | Fatty acid biosynthesis | ydbS-ydbT-S160-S162-acpS | Yes | | Secondary $σ^W$ |
potentially false negative exclusions, we maintained all the genes that were down-regulated with q-values higher than 0.05 but p-values lower than 0.05 also in our shortlist of potentially σW-regulated genes. These genes were further analyzed by assessing their transcription profiles under 104 conditions, including several conditions known to induce SigW.

Definition of the σW regulon by assessment of transcript profiles across conditions

To minimize the false positive identifications of σW-regulated genes, we took advantage of a large-scale tiling array analysis of gene expression in B. subtilis 168 across 104 conditions, involving 269 hybridizations [35]; GEO accession number GPL15150]. Within this previous study promoters of different sigma factors were classified based on an unsupervised algorithm. Notably, σW regulated promoters were classified together with the other ECF sigma factors (σE, σX, σY and σM) as having sigma factor binding sites of the ‘σWXXY’ type, because no distinction between promoters recognized by sigma factors with similar DNA binding motifs could be made (note that although this binding site was annotated as ‘σWXXY’, it also covers the σM binding site). Importantly, the results of this study revealed marked differences in the transcription profiles of the sigW, sigX, sigY and sigM genes across conditions, especially during heat, salt and ethanol stress (Figure 2). This was an important observation, because it can help in the dissection of the respective regulons. The analysis of transcription profiles across the 104 conditions showed that the transcription profiles of 59 genes cluster with that of sigW in the previously defined transcription cluster C9 (Figure 3, [35]). Importantly, most genes in cluster C9 were found to be significantly down-regulated in the sigW mutant in our present studies and/or were previously reported as σW-regulated (Figure 4). The 12 genes within cluster C9 that are not σW-regulated represent members of the σX regulon, including the sigI gene itself. Their presence in cluster C9 relates to the fact that σX-regulated genes behave quite similarly to σW-regulated genes, the main distinguishing feature being that they are induced by ethanol stress rather than salt stress. Clearly, the known σX-regulated genes in cluster C9 were not down-regulated in the sigW mutant, whereas all other genes in cluster C9 were down-regulated in the sigW mutant (Figure 4A). Only one gene in cluster C9, yxcE, which was previously reported to be σX-regulated, did not qualify as a σX-regulated gene in our statistical analyses as its down-regulation in the sigW mutant (effect value −0.45) had a p-value of 0.08. However, based on the combined data, we believe that yxcE should be regarded as a member of the σX regulon. Accordingly, the long 3’ UTR of yxcE with the designations S1489 is probably also part of the σW regulon, which is supported by the fact that it is present in cluster C9 (Table 3).

Table 2. Cont.

| Name | Effect | Function | Regulators | Genetic organization | Cluster | σWXXY promoter sequence | Conclusion |
|------|--------|----------|------------|----------------------|---------|-------------------------|------------|
| sigW | −0.44  |          |            |                      |         |                         |            |
| ydcC | −0.42  | σW      | ydcC       | C2                   | Yes     | Secondary σW            |            |
| thiC | −0.41  | Thiamine biosynthesis | Thi-box | Downstream of ygiA | C48     | No Background            |            |
| ydoF | −0.41  | σW, σE  | yeaA-ydp-ydoF | C2 | Yes | Secondary σW |            |
| yocM | −0.41  |          |            |                      |         |                         |            |

Only the down-regulated genes with effect values lower than −0.4 and p-values lower than 0.05 are shown. Effect values marked with * have q-values of less than 0.05. For each individual gene, the Table lists the function, the previously identified regulation, the genetic organization, the condition-dependent transcription profile cluster as defined by Nicolas et al. (35), the presence of a predicted ‘σWXXY’, promoter sequence (35), and our conclusion whether it belongs to the σW core regulon or the secondary σW-regulated genes. It should be noted here that the previously predicted ‘sigmaWXXY’ promoter sequence (35) also covers the potential binding site for σW. The division between group 1 and group 2 genes is indicated by a bold line.

doi:10.1371/journal.pone.0048471.t002

For each individual gene, the Table lists the function, the previously identified regulation, the genetic organization, the condition-dependent transcription profile cluster as defined by Nicolas et al. (35), the presence of a predicted ‘σWXXY’, promoter sequence (35), and our conclusion whether it belongs to the σW core regulon or the secondary σW-regulated genes. It should be noted here that the previously predicted ‘sigmaWXXY’ promoter sequence (35) also covers the potential binding site for σW. The division between group 1 and group 2 genes is indicated by a bold line.
### Table 3. Previously Reported σ^{W}-regulated genes that were not significantly down-regulated in the sigW mutant strain.

| Name       | Effect sigW/WT | Function                          | Regulation       | Genetic organization | Cluster | σ^{WXY} promoter sequence | Conclusion          |
|------------|----------------|----------------------------------|------------------|----------------------|---------|--------------------------|---------------------|
| yxzE       | −0.45          | Iron uptake                      | σ^{W}, σ^{A}     | yxzE-S1489            | C9      | Yes                      | core σ^{W}          |
| S1489      | −0.31          | Iron uptake                      | σ^{W}, σ^{A}     | yxzE-S1489            | C9      | Yes                      | core σ^{W}          |
| bsdR (fatR) | −0.15          | Fatty acid biosynthesis           | σ^{M}, σ^{W}, σ^{A} | ythH-bsdR-yrhJ       | C10     | Yes                      | σ^{A}               |
| ywhO       | −0.02          | Iron uptake                      | σ^{W}, σ^{A}     | ywhB-ywhO            | C29     | No                       | Not σ^{W} - Fur-regulated |
| fabHα      | −0.03          | Fatty acid biosynthesis           | σ^{W}, FapR      | fabHα-fabF           | C3      | Yes (P5 +114)            | σ^{W} Kingston et al 2011 |
| efeN (ywbN) | −0.04          | Iron uptake                      | σ^{W}, σ^{A}     | ywbL-ywbM-ywbN ywbN-ywbO | C29 | No                       | Not σ^{W} - Fur-regulated |
| fabF       | −0.07          | Fatty acid biosynthesis           | σ^{W}, FapR      | fabHα-fabF           | C3      | Yes                      | σ^{W} Kingston et al 2011 |
| yrhJ (cypB) | −0.08          | Fatty acid biosynthesis           | σ^{W}, σ^{A}     | yrhJ-ythH-yrhJ       | C10     | Yes                      | σ^{W}               |
| ywaC       | 0.06           | (p)pGpp synthetase                | σ^{W}, σ^{A}     | ywaC-spx             | C79     | Yes                      | σ^{M}               |
| yjbC       | 0.07           | PerR, σ^{W}, σ^{A}               | yjbC-spx         | yjbC-spx             | C5      | Yes                      | σ^{A} and σ^{M}     |
| yjbD (spxA) | 0.17           | PerR, σ^{W}, σ^{A}               | yjbC-spx         | yjbC-spx             | C17     | Yes                      | σ^{A} and σ^{M}     |
| divIC      | 0.23           | Septum formation                 | σ^{W}, σ^{A}     | divIC-yabO           | C20     | Yes                      | σ^{M}               |
| yrhH       | 0.25           | Gene regulation during transition phase | σ^{W}, σ^{A}   | yrhH-ythH-yrhJ       | C10     | Yes                      | σ^{M}               |
| abh        | 0.30           | Gene regulation during transition phase | σ^{W}, σ^{A}   | yrhH-ythH-yrhJ       | C36     | Yes                      | σ^{A}               |
| ywnJ       | 0.31           | Gene regulation during transition phase | σ^{W}, σ^{A}   | yrhH-ythH-yrhJ       | C2      | Yes                      | σ^{A}               |
| bcrC       | 0.33           | Resistance to bacitracin and oxidative stress | σ^{W}, σ^{A}   | yrhH-ythH-yrhJ       | C10     | Yes                      | σ^{A}               |
| yglJ       | 0.37           | Resistance against paraquat       | σ^{W}, σ^{A}     | yrhH-ythH-yrhJ       | C78     | Yes                      | σ^{A}, σ^{M}        |

For each individual gene, the Table lists the function, the previously identified regulation, the genetic organization, the condition-dependent transcription profile cluster as defined by Nicolas et al [35], the presence of a predicted σ^{WXY} promoter sequence [35], and our conclusion whether it belongs to the σ^{A} core regulon or the secondary σ^{W}-regulated genes. It should be noted here that the previously predicted σ^{WXY} promoter sequence [35] also covers the potential binding site for σ^{M}.

[doi:10.1371/journal.pone.0048471.t003]
for example, S740, S739 and yucM are all down-regulated in the sigW mutant and induced upon salt stress (Figure 5A). Similarly, downstream of the ydbST operon, S161, acpS and S162 are down-regulated in the sigW mutant and induced upon salt stress (Figure 5B). In other cases the situation is different. For example, ygzA, a gene starting close to the start site of spo0M, but running in the opposite direction, is also down-regulated in the sigW mutant. Nevertheless, ygzA is not preceded by a consensus binding sequence for σWXY, and this gene is also not induced by salt stress. Likewise, the yxjH gene downstream of the σW-regulated gene yxjI is down-regulated in the sigW mutant, but also in this case no induction is observed during salt stress. Thus, we do not consider ygzA and yxjH to be genuinely σW-regulated genes.

15 genes that were previously reported to be σW-regulated were not down-regulated in the sigW mutant (Table 3, Figures 1, 3 and 4). This observation cannot be explained by a simple absence of expression of these genes in the parental strain that would have precluded the possibility to observe their down-regulation. This view is supported by the finding that the distribution of the expression levels of these genes in the parental strain was not significantly different from the distribution of the expression levels of genes belonging to groups 1 and 2 (Mann-Whitney U-test p-value of 0.64). Indeed, these genes have been assigned to multiple σ regulons besides the σW regulon and they mostly appear to show condition-dependent transcription profiles that are more similar to those of genes regulated by σ factors other than σW (Table 3). We therefore examined whether these genes had been previously shown to act in a typical σW-dependent manner, or whether their dependency on other ECF sigma factors had been shown (Table S1). The majority of these 15 genes do not show a typical upregulation pattern under conditions inducing the σW regulon. 11 of the 15 genes have been shown to be regulated by other sigma factors (10 by σM and 1 by σN). ywaJ, ywbN and ywhH have only been shown to have the potential for binding σW in vitro [7,8,25], and no in vivo data suggest a σW-dependence of their promoters. fabHa has been shown to be expressed σW-dependently [31], and upregulation of the fabHa-fabF operon has been reported upon overexpression of σW [2]. However, this operon was never observed to be upregulated in any of the conditions known to induce the σW regulon. This is somewhat surprising, but may be explained by the promoter being located within the fabHa gene itself. The majority of these 15 genes are therefore unlikely to be σW-regulated.

Lastly, 40 genes appeared to be up-regulated in the sigW mutant with effect values of more than 0.4 and p-values of less than 0.05 (Table 4). However, it should be noted that none of these changes have q-values smaller than 0.05. This suggests that these up-regulations may represent false positive results or indirect effects that are not as strong as direct regulatory effects. Several of the up-regulated genes are located in the close proximity of σW-regulated genes, but are encoded by the opposite strand. Two of these genes, ybbK and ybbF, are located immediately opposite of sigW and,

Figure 2. Expression profiles of sigW, sigX, sigY and sigM in B. subtilis 168 across 104 conditions. The 269 tiling array hybridizations [35] are arranged along the x-axis. Of particular interest for discriminating the activities of the encoded sigma factors are the conditions heat stress (‘heat’), ethanol stress (‘etha’) and hypersaline stress (‘salt’), which are marked by pink shading.
doi:10.1371/journal.pone.0048471.g002
therefore, the up-regulation of these genes in the sigW mutant could be the result of a polar effect of the deletion of sigW. However, the transcription profiles of both of these genes do not show changes during exposure to high salt and the same is true for the other up-regulated genes. Therefore, we do not consider ybbK, ybbJ and other genes up-regulated in the sigW mutant as novel sigW-regulated genes.

Function of the sigW regulon

The sigW regulon is responsible for activating genes whose products are likely to be needed upon envelope stress, or beneficial under conditions of alkali shock, salt stress and treatment with cationic peptides and agents that impair cell wall biosynthesis [9,18,30,39,43]. To verify this view, the genes identified in our study as being part of the sigW regulon were analysed for function according to their classification in SubtiWiki [17] (Table 2, Table S1). Indeed, the groups of genes that were most largely represented encoded cell envelope stress proteins, membrane proteins and proteins involved in resistance against toxins or antibiotics. These proteins have been implicated in protecting the cell from stresses that affect the membrane and in detoxification upon contact with toxic compounds. Our present findings suggest that, also under non-stress conditions, it may be beneficial for B. subtilis to express the respective sigW-regulated genes at a basal level, for example to allow fast and effective responses to any membrane stresses that may suddenly occur. Notably, over half of the genes identified as being sigW-regulated are B. subtilis ‘y’ genes, essentially genes that have yet to be functionally annotated. Therefore, until the functions of these genes are defined it will remain difficult to determine which sigW-regulated genes function in what capacity when the regulon is upregulated.

Comparison of global transcription in rasP,prsW and sigW mutant cells

Deletion of the genes for RasP and PrsW under stress conditions inhibits the activation of the sigW-regulon, because both of these proteases are required for inactivation of the sigW anti-sigma factor RsiW. Thus, no activation of sigW-controlled genes was detectable in rasP or prsW mutant cells upon stress [15,19,21,42]. In addition, the rasP mutant is known to display several phenotypes, such as defects in competence and protein secretion, which are not observed in prsW or sigW mutants [20,32,41,47]. During membrane protein overproduction, the rasP mutant also behaves differently from the prsW and sigW mutants. Whereas prsW and sigW mutations generally improve membrane protein overproduction, in the rasP mutant overproduction of all tested membrane proteins was abolished [50].

We wanted to know whether RasP and PrsW, the genes of which are both expressed under the tested non-stress conditions, play a role in the control of the basal activity of the sigW regulon. Generally, the transcriptional changes in the rasP or prsW mutant strains compared to the parental strain and the sigW mutant were rather small and only few had q-values below 0.05 (15 in the rasP/WT comparison, 0 in the prsW/WT comparison, 21 in the rasP/sigW comparison, and 14 in the prsW/sigW comparison). Closer examination revealed that only 3 genes associated with q-values below 0.05 were not predicted to belong to the global ECF regulon defined in Nicolas et al. [35] (i.e. natA, hisG and tedB). We therefore reasoned that statistical power could be increased by searching for
The Bacillus SigW Regulon

differential expression in priority among the 243 genes and new expression segments included in this analysis that were previously classified as members of the global ECF regulon [35]. Indeed, the estimates that we obtained for the false discovery rates of global ECF regulon genes with p-values ≤ 0.05 were 9.7% for the sigW/WT comparison, 10.5% for the rasP/WT comparison, 12.3% for the prsW/WT comparison, 11.8% for the rasP/sigW comparison and 13.4% for the prsW/sigW comparison. These genes are listed in Tables S2, S3, S4. For completeness, other genes with p-values ≤ 0.05 have also been listed although they probably include a much higher fraction of false discoveries. Altogether, the composition of these lists revealed that the afore-described σ^W-regulated genes were down-regulated in both the rasP and prsW mutants, but to lesser extents than in the sigW mutant. This indicates that the deletion of rasP or prsW indeed decreased the activity of σ^W, but that σ^W activity not completely abolished in the respective mutants under the applied non-stress conditions (Figure 6, Table S2A). Apparently, some σ^W molecules were able to escape from binding to RasW, even in the absence of RasP or PrsW, thereby causing low-level expression of the σ^W regulon that was independent from intramembrane proteolysis by RasP and PrsW. Among the non-σ^W-regulated genes that were down-regulated in the rasP mutant were several genes that are involved in the development of genetic competence (i.e. oppA, nucA, ssbB, rnpA). Other genes that were specifically down-regulated in the sigW mutant mainly relate to lipid and cell wall turnover.

In both the rasP and prsW mutant strains, slight increases in transcription were detected for genes involved in compatible solute transport, which is important for osmoregulation (Table S2). Even though not all of these genes were always significantly up-regulated in each mutant, there seemed to be a mild, general up-regulation of these genes in both the rasP and prsW mutant strains. Additionally, slightly increased transcription of genes involved in teichoic acid synthesis, phospholipid biosynthesis, cell wall biogenesis and cell shape was observed. Genes that were specifically up-regulated in the rasP mutant include genes involved in amino acid metabolism (e.g. genes for histidine and arginine biosynthesis, and ornithin and citrullin utilization) and genes involved in cell envelope stress systems (e.g. the natAB-yeck operon [11,36,37], the LiaRS, WalRK [4,13] and DesRK two-component systems, and the σ^M-regulon [14,28,34]). However, not all genes regulated by these systems were up-regulated and therefore the significance of these findings remains unclear.

Notably, in our previous studies we have reported significantly increased levels of HtrA and HtrB in the rasP mutant [50]. Nevertheless, the cssS and css transcription levels were only slightly down-regulated in the rasP mutant and the same was true for the sigW or prsW mutant strains (Table 5). Furthermore, the transcription of the CsSRS-regulated htrA and htrB genes was not significantly altered in rasP, prsW or sigW mutant cells (Table 5). This implies that the activity of the CsSRS system is not responsible for the increased HtrA and HtrB levels in the rasP mutant.

Lastly, a direct comparison of global transcription in the rasP and sigW mutant strains resulted in very few statistically significant changes (Tables S3 and S4). Compared to the sigW mutant, a few...
Table 4. Genes that were up-regulated in the *sigW* mutant.

| Name   | Effect sigW/WT | Function                                      | Regulation                  | Genetic organization | Cluster |
|--------|----------------|-----------------------------------------------|-----------------------------|----------------------|---------|
| ybbK   | 3.07           | ybbK-ybbJ Opposite of *sigW* (↑)               |                             |                      | C6      |
| ybbJ   | 2.68           | ybbK-ybbJ Opposite of *sigW* (↑)               |                             |                      | C6      |
| S928 (inter) | 2.25         | Between *mgsR* and *nsbRD*                     |                             |                      | C5      |
| S1380  | 1.16           |                                               |                             |                      | C10     |
| yzkV   | 1.13           |                                               |                             |                      | C2      |
| S1026 (inter) | 0.92          | Upstream of *yrzI* (↑)                          |                             |                      | C2      |
| cotT   | 0.91           |                                               |                             |                      | C2      |
| yodl   | 0.83           | σ^K                                           |                             |                      | C2      |
| S1030  | 0.82           | 5’ of *yhrF*,                                 |                             |                      | C3      |
| murG   | 0.77           | Peptidoglycan precursor biosynthesis           | σ^K, σ^M,Spo0I              |                      | C4      |
| S981   | 0.73           | 3’ of *yapP*, opposite of *yqfR* (↑) and *yqbc* (↑) |                             |                      | C17     |
| ymoG   | 0.68           | Spore coat protein                             | σ^K                         |                      | C2      |
| S655   | 0.66           | Opposite of *fasB* (↓)                         |                             |                      | C17     |
| S682   | 0.65           | 5’ of *spolVA*                                 |                             |                      | C2      |
| S1356  | 0.64           | 5’ of *degS* (↑)                               |                             |                      | C3      |
| yrzI   | 0.63           |                                               |                             |                      | C2      |
| S613   | 0.62           | 5’ of *yraZD* (slightly ↑)                     |                             |                      | C27     |
| S663   | 0.61           | 5’ of *cdxA* (slightly ↑)                      |                             |                      | C17     |
| S1405 (inter) | 0.60          | Downstream of *spolID* (slightly ↑)            |                             |                      | C2      |
| S254 (indep) | 0.60         |                                               |                             |                      | C17     |
| yzkV   | 0.59           | RNA that inhibits AhrC translation              | CcpN regulon                |                      | C30     |
| S653 (indep) | 0.57           | Downstream of *fasB* (↓)                        |                             |                      | C17     |
| ydeH   | 0.56           | AbrB                                          |                             |                      | C17     |
| yqarT  | 0.54           | Close to S981 (↑) and *yqbC* (↑)               |                             |                      | C6      |
| S360 (inter) | 0.54           |                                               |                             |                      | C35     |
| S118 (inter) | 0.52           | Opposite of *yuaI-yuaF-yuaG*                   |                             |                      | C52     |
| obg    | 0.50           | Ribosome assembly (essential), possibly required for Spo0A-activation |                             |                      | C3      |
| cotU   | 0.50           | Spore coat protein                             | GerE, GerR                  |                      | C2      |
| yapD   | 0.46           | σ^K                                           | upstream of S952 (slightly ↑) |                      | C153    |
| S278   | 0.46           | 5’ *yfzA* (↑)                                  |                             |                      | C17     |
| pssA   | 0.46           | Biosynthesis of phospholipids                   |                             |                      | C3      |
| S303   | 0.45           | 5’ of *ygaA*                                  |                             |                      | C3      |
| comK   | 0.44           | Competence and DNA uptake regulation            | AbrB, ComK, DegU, CodY, Rok |                      | C1      |
| yktD   | 0.43           |                                               |                             |                      | C115    |
| S1543 (intra) | 0.43          | Upstream of *yydL* and *yadJ* (both slightly ↑) |                             |                      | C35     |
| S95    | 0.42           | 5’ of *ycbI*                                  |                             |                      | C35     |
| S831   | 0.42           | 5’ of *ypeP*                                  |                             |                      | C2      |
| S427   | 0.42           | 5’ of *yjzE*                                  |                             |                      | C2      |
| S924   | 0.41           | 5’ of *sinI*                                  |                             |                      | C17     |
| yfzA   | 0.41           |                                               |                             |                      | C17     |

Only the genes with Effect values higher than 0.4 and p-values lower than 0.05 are shown. Arrows behind genes in the ‘genetic organization’ column indicate whether the transcription of these genes was up- (↑) or down-regulated (↓). For each individual gene, the Table lists the function, the previously identified regulation, the genetic organization, and the condition-dependent transcription profile cluster as defined by Nicolas et al [35].

doi:10.1371/journal.pone.0048471.t004
genes including rocD and rocA, natA and natB, des and argI were specifically up-regulated in the rasP mutant. Other transcriptional changes summarized in Table S3A relate to changes in the sigW mutant. For the genes that were down-regulated in the rasP mutant, most hits were specific for the rasP mutant. No clear pattern however emerges from these changes, although some of these genes relate to the cell envelope metabolism (membrane and cell wall). Furthermore, the vast majority of genes found to be differentially expressed in the prsW mutant compared to the sigW mutant relate to σW-regulated genes. Only the up-regulation of the pstS, pstD, pstBB, pstA and psbC genes for phosphate uptake was very specific for the prsW mutant. The reasons for these specific differences in transcription in the rasP, prsW or sigW mutant strains remains to be determined.

Discussion

The σW-regulon has been extensively described in several previous papers, and 69 genes have been reported as σW-controlled genes [8,9,25,26,31]. However, it has so far remained very difficult to discriminate between genes of the σW-regulon and the other ECF σ-regulons of B. subtilis, as the respective promoter sequences and the stress stimuli for induction partially overlap [9,14,25]. Indeed, in the study reporting the transcriptional profile of B. subtilis grown in 104 conditions [35], only a global ECF sigma factor regulon was described, and no clear definition of the σW regulon could be generated. Also, it was so far unknown which mRNAs of B. subtilis are part of the σW-regulon. In our present studies, we have therefore employed tiling array data to define the transcriptome of a sigW mutant B. subtilis strain. Then the results were examined in the light of the recently described transcriptome of the parental strain 168 across 104 different conditions [35]. Our results show that 89 genes of B. subtilis are regulated by σW and the data suggest that 13–15 of the 69 previously reported σW-regulated genes might represent false-positive identifications. In addition to 53 already known σW-regulated genes, we have discovered 36 novel genes of the σW-regulon and we found that several σW-regulated operons are larger than initially thought.

Two subgroups of σW-regulated genes can be discerned based on the effect values for their down-regulation in sigW mutant cells. This differential down-regulation pattern does not correlate with the expression levels of these genes in the parental strain. However, there appears to be a bias for genes that are located at the downstream ends of certain large operons that often have low effect values (i.e. group 2 genes), whereas the genes located more upstream in these operons tend to have high effect values (group 1 genes). On the other hand, several complete operons display high effect values from start to end, while other complete operons have low effect values from start to end. This indicates that the location of a gene in an operon can influence whether it belongs to group 1 or group 2. However, it remains to be determined which additional mechanisms are responsible for the observed bimodal pattern in σW regulation. Another novel finding was that several apparently non-σW-regulated genes on the opposite strand of σW-regulated genes turned out to be slightly up-regulated in the sigW mutant. This indicates that the transcriptional activity of σW-regulated genes can have a negative impact on the transcription of genes encoded by the opposite strand. The molecular basis for this effect is currently not known. However, it is conceivable that RNA-polymerase initiating with σW may directly or indirectly dampen the transcription elongation efficiency of RNA-polymerase transcribing into the opposite direction.

As expected, the σW-regulated genes were also down-regulated in rasP or prsW mutant strains, albeit to lesser extents than in the sigW mutant. This implies that there is residual σW activity in the absence of either the RasP or PrsW proteases, which may relate to the equilibrium between the free states of σW plus RsiW and the σW-RsiW bound state. Such leakiness is not an uncommon feature among biological systems. Alternatively, certain other proteases may also be capable of degrading limited amounts of RsiW in the absence of RasP or PrsW. Candidate proteases for alternative

Table 5. Transcriptional changes of genes regulated by the CssRS two-component system.

| Gene | Effect sigW/WT | Effect rasP/WT | Effect prsW/WT |
|------|----------------|----------------|----------------|
| cssR | -0.20          | -0.20          | -0.19          |
| cssS | -0.22          | -0.22          | -0.03          |
| htrA | -0.24          | 0.21           | 0.07           |
| htrB | -0.29          | 0.18           | 0.06           |

doi:10.1371/journal.pone.0048471.t005

Figure 6. Up- and down-regulation of genes in rasP, prsW or sigW mutant strains compared to the wild-type. A, Venn diagram for down-regulated genes. B, Venn diagram for upregulated genes. Only genes with transcriptional changes that have p-values lower than 0.05 and effect values lower than −0.40 (A) or higher than 0.40 (B) are included. The genes that are considered to be σW-regulated are indicated between brackets.
doi:10.1371/journal.pone.0048471.g006
RsiW cleavage in the absence of PrsW might be the membrane-bound forms of HrA and HrB. Both HrA and HrB are closely related to the site-I protease DegS of E. coli, which has been implicated in RIP of the anti-sigma factor RseA that sequesters $\sigma^C$ [1,12,30]. It should be noted that, compared to the previously used methods for assessing the effects of mutations in rasP or $\text{prsW}$ [15,19,21,42], the presently performed tiling array analyses are more sensitive and they can reproducibly reveal smaller changes. This is probably the reason why residual $\sigma^W$ activity in the absence of RasP or PrsW has so far remained unnoticed.

In relation to the previously documented defects of rasP mutant cells in competence development [20,32], protein secretion [32,41], and membrane protein overproduction [50], we verified whether any of these defects could be connected to transcriptional changes. However, as indicated above, the observed transcriptional changes in the rasP mutant were generally very minor and, apart from four competence-related genes, no changes were found that might explain any of the observed phenotypes through transcriptional regulation. The four affected competence-related genes ($\text{msxl}$, $\text{opfA}$, $\text{ssbB}$ and $\text{ypdD}$) were only very slightly down-regulated in the rasP mutant and this finding should be viewed with caution, because the present analyses were performed with cells grown in LB medium, which is not an optimal medium for inducing competence. Taken together, we conclude that the observed defects of rasP mutant cells in protein secretion and membrane protein overproduction most likely relate to post-transcriptional regulatory mechanisms that would involve the enzymatic activity of the RasP protease. However, we cannot completely exclude the possibility that changes in the membrane fluidity contribute to the pleiotropic phenotype of rasP mutant cells. This relates to recent studies by Kingston et al., 2011 [31], who proposed that activation of a $\sigma^W$-dependent promoter in the $\text{fabHa-fabF}$ operon results in a higher proportion of straight-chain fatty acids and a longer average chain length in phospholipids, which will cause a reduced fluidity of the membrane. It should be noted however that under non-stress conditions we observed no influence of the absence of $\sigma^W$ on the expression of $\text{fabHa}$.

In conclusion, the present studies lead to a definition of the $\sigma^W$ regulon under non-stress conditions (exponential growth in LB broth at $37^\circ C$) that have been applied in numerous studies over the past decades. Importantly, the use of non-stress conditions allowed us to determine the basal expression levels of $\sigma^W$-regulated genes, and to avoid side effects of particular stresses on the entire regulatory network of the cell. By following this strategy, we have considerably reduced the complexity of the system, which permitted us (i) to pinpoint the most strictly $\sigma^W$-dependent genes that probably have promoter sequences with the highest affinity for $\sigma^W$, and (ii) to classify the known and newly identified $\sigma^W$-controlled genes. Furthermore, our studies provide novel insights in the importance of the RIP proteases PrsW and RasP in the activation of this stress-responsive regulon. Especially, the observation that the absence of either PrsW or RasP does not lead to a complete inactivation of $\sigma^W$-dependent gene expression is intriguing and calls for further investigations. Although this expression is most likely caused by an equilibrium where low levels of $\sigma^W$ bind to RNAP instead of the anti-sigma factor RsiW, it cannot be excluded that certain, so far unknown, signals trigger alternative pathways for RsiW inactivation, or that PrsW and RasP might be substituted to some extent by other proteases. Lastly, our present findings strongly support the view that RasP is not only directly involved in the activation of the $\sigma^W$-regulon, but also in other post-transcriptional regulatory mechanisms relating to competence development, protein secretion and membrane protein biogenesis.

Supporting Information

Table S1 Previously identified $\sigma^W$-regulated genes. (XLSX)

Table S2 Genes down- or up-regulated in $\text{sigW}$, $\text{prsW}$ or rasP mutant strains. Changes associated with p-values<0.05 are indicated in bold. A, down-regulated genes. B, up-regulated genes. (DOCX)

Table S3 Genes up- or down-regulated in the rasP mutant strain compared to the $\text{sigW}$ mutant strain. Changes associated with p-values<0.05 are indicated in bold. A, up-regulated genes. B, down-regulated genes. (DOCX)

Table S4 Genes up- or down-regulated in the $\text{prsW}$ mutant strain compared to the $\text{sigW}$ mutant strain. Changes associated with p-values<0.05 are indicated in bold. A, up-regulated genes. B, down-regulated genes. (DOCX)

Acknowledgments

The authors thank Ulrike Mader for helpful discussions and support.

Author Contributions

Conceived and designed the experiments: JCZ, JMD ELD. Performed the experiments: JCZ ELD. Analyzed the data: JCZ PN ELD. Contributed reagents/materials/analysis tools: TW JMD. Wrote the paper: JCZ PN TW JMD ELD.

References

1. Alba BM, Leech JA, Oaufryk C, Lu CZ, Gross CA (2002) DegS and YaeL participate sequentially in the cleavage of RseA to activate the sigmaE-dependent extracytoplasmic stress response. Genes Dev 16: 2156–2160.

2. Asai K, Yamasuichi H, Kang CM, Yoshida K, Fujito Y, et al (2005) DNA microarray analysis of Bacillus subtilis sigmas factors of extracytoplasmic function family. FEMS Microbiol Lett 220: 155–160.

3. Strimmer K (2000) A unified approach to false discovery rate estimation. BMC Bioinformatics 9: 303.

4. Buschiazzo P, Lioudi E, Noone D, Salzberg LI, Botella E, et al (2010), Peptidoglycan metabolism is controlled by the WalRK, YveFG and PhoPR two-component systems in phosphate limited Bacillus subtilis cells. Mol Microbiol 75: 972-993.

5. Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19: 185–193.

6. Butcher BG, Helmann JD (2006) Identification of Bacillus subtilis sigmab-dependent genes that provide intrinsic resistance to antimicrobial compounds produced by Bacilli. Mol Microbiol 60: 765–782.

7. Cao M, Helmann JD (2004) The Bacillus subtilis extracytoplasmic-function sigmaX factor regulates modification of the cell envelope and resistance to cationic antimicrobial peptides. J Bacteriol 186: 1136–1146.

8. Cao M, Kober PA, Morschheider MM, Wu MF, Paddon C, et al (2002) Defining the Bacillus subtilis sigma(W) regulon: a comparative analysis of promoter consensus search, run-off transcription/microarray analysis (ROMA), and transcriptional profiling approaches. J Mol Biol 316: 443–457.

9. Cao M, Wang T, Ye R, Helmann JD (2002), Antibiotics that inhibit cell wall biosynthesis induce expression of the Bacillus subtilis sigma(W) and sigma(M) regulons. Mol Microbiol 45: 1267–1276.

10. Chen G, Zhang X (2010) New insights into S2P signaling cascades: regulation, variation, and conservation. Protein Sci 19: 2015–2020.

11. Cheng J, Guffanti AA, Kubruch TA (1997) A two-gene ABC-type transport system that extrudes Na+ in Bacillus subtilis is induced by ethanol or propanolone. Mol Microbiol 23: 1107–1120.

12. Dolbey RE, Wang P, van Dijl JM (2012) Membrane proteins in the bacterial protein secretion and quality control pathway. Microbiol Mol Biol Rev 76: 311–330.
13. Dubhar S, Biscia D, Devine K, Maeder T (2008) A matter of life and death: cell wall homeostasis and the WallKR (YrGRF) essential signal transduction pathway. Mol Microbiol 70: 1307–1322.
14. Eiamphuangwor W, Helmann JD (2006) The Bacillus subtilis sigmaM regulon and its contribution to cell envelope stress responses. Mol Microbiol 67: 830–848.
15. Elmerriex CD, Losick R (2006) Evidence for a novel protease governing regulated intramembrane proteolysis and resistance to antimicrobial peptides in Bacillus subtilis. Gene Dev 20: 1911–1922.
16. Eymann C, Homuth G, Scharf C, Becker M (2002) Bacillus subtilis functional genomics: global characterization of the stringent response by proteome and transcriptome analysis. J Bacteriol, 184: 2500–2520.
17. Florez LA, Rapol SE, Schmeisky AG, Lammers CR, Stulke J (2009) A community-curated consensus annotation that is continuously updated: the Bacillus subtilis centred wiki SubtiWiki, Database (Oxford): ba0112.
18. Halen H, Mader U, Otto A, Bons F, Steil L, et al (2010) A comprehensive proteomics and transcriptomics analysis of Bacillus subtilis salt stress adaptation. J Bacteriol 192: 870–882.
19. Heinrich J, Hein K, Wiegert T (2009) Two proteolytic modules are involved in regulated intramembrane proteolysis of Bacillus subtilis RsiW. Mol Microbiol 74: 1412–1426.
20. Heinrich J, Lundén T, Kontinen VP, Wiegert T (2008) The Bacillus subtilis ABC transporter EcsAB influences intramembrane proteolysis through RasP. Microbiology 154: 1989–1997.
21. Heinrich J, Wiegert T (2006) YtaC determines site-1 degradation in regulated intramembrane proteolysis of the RsiW anti-sigma factor of Bacillus subtilis. Mol Microbiol 62: 566–579.
22. Heinrich J, Wiegert T (2009) Regulated intramembrane proteolysis in the control of extracytoplasmic function sigma factors. Res Microb 160: 696–705.
23. Helmann JD (2002) The extracytoplasmic function (ECF) sigma factors. Adv Microb Physiol 46: 47–110.
24. Ho TD, Ellermeier CD (2012) Extra cytoplasmic function sigma factor activation. Curr Opin Microbiol 15: 182–186.
25. Huang X, Fredrick KL, Helmann JD (1998) Promoter recognition by Bacillus subtilis sigW: autoregulation and partial overlap with the sigmaX regulon. J Bacteriol 180: 3763–3770.
26. Huang X, Gaballa A, Cao M, Helmann JD (1999) Identification of target promoters for the Bacillus subtilis extracytoplasmic function sigma factor, sigma W. Mol Microbiol 31: 361–371.
27. Hughes KT, Matthe K (1996) The anti-sigma factors. Ann Rev Microbiol 50: 231–286.
28. Jervis AJ, Thackray PD, Houston CW, Horsburgh MJ, Moir A (2007) SigM-dependent stress response in Bacillus subtilis that reduces membrane fluidity. J Bacteriol 189: 6919–6927.
29. Jordan S, Hutchings MI, Mascher T (2008) Cell envelope stress response in Gram-positive bacteria. FEMS Microbiol Rev 32: 107–146.
30. Karcher K, Ito K, Akiyama Y (2002) YtaC, (EcsE) activates the sigmaE pathway of stress response through a site-2 cleavage of anti-sigmaE, Rock. Genes Dev 16: 2147–2155.
31. Kingstone AW, Subramanian C, Rock CO, Helmann JD (2011) A sigmaW-dependent stress response in Bacillus subtilis that reduces membrane fluidity. Mol Microbiol 81: 69–79.
32. Leskela S, Kontinen VP, Sarvas M (1996) Molecular analysis of an operon in Bacillus subtilis encoding a novel ABC transporter with a role in exoprotein production, sporulation and competence. Microbiology 142: 71–77.
33. Luo Y, Helmann JD (2009) Extracytoplasmic function sigma factors with overlapping promoter specificity regulate sublancin production in Bacillus subtilis. J Bacteriol 191: 4951–4958.
34. Macshe T, Haschmann AB, Helmann JD (2007) Regulatory overlap and functional redundancy among Bacillus subtilis extracytoplasmic function sigma factors. J Bacteriol 190: 6919–6927.
35. Nicolas P, Mader U, Deryn E, Rochat T, Leduc A, et al (2012) Condition-dependent transcriptome reveals high-level regulatory architecture in Bacillus subtilis. Science 335: 1103–1106.
36. Ogura M, Tsukahara K, Hayashi K, Tanaka T (2007) The Bacillus subtilis NalK-NarL two-component system regulates expression of the narAB operon encoding an ABC transporter for sodium ion extrusion. Microbiology 153: 667–675.
37. Padan E, Schuldiner S (1994) Molecular physiology of Na+/H+ antiporters, key transporters in circulation of Na+ and H+ in cells. Biochim Biophys Acta 1185: 129–151.
38. Petersen A, Bragdulla M, Haas S, Hoheisel JD, Volker U, et al (2001) Global analysis of the general stress response of Bacillus subtilis. J Bacteriol 183: 5617–5631.
39. Pietiaenen M, Gardemeister M, Mecklin M, Leskela S, Sarvas M, et al (2005) Cationic antimicrobial peptides elicit a complex stress response in Bacillus subtilis that involves ECF-type sigma factors and two-component signal transduction systems. Microbiology 151: 1527–1539.
40. Rasmussen S, Nielsen HB, Jarmer H (2009) The transcriptionally active regions in the genome of Bacillus subtilis. Mol Microbiol 73: 1043–1057.
41. Saito A, Hizukuri Y, Matsu T, Chiba S, Mori H, et al (2011) Post-liberation cleavage of signal peptides is catalyzed by the site-2 protease (S2P) in bacteria. Proc Natl Acad Sci U S A 108: 13740–13745.
42. Schobel S, Zellmer S, Schumann W, Wiegert T (2004) The Bacillus subtilis sigmaW anti-sigma factor RsiW is degraded by intramembrane proteolysis through YtaC. Mol Microbiol 52: 1109–1119.
43. Steil L, Hofmann T, Budde I, Volker U, Bremer E (2003) Genome-wide transcriptional profiling analysis of adaptation of Bacillus subtilis to high salinity, J Bacteriol 185: 6358–6370.
44. Sterber S, del Peso-Sanzos T, Shingler V (2011) Regulation of alternative sigma factor use. Annu Rev Microbiol 65: 37–55.
45. Tojo S, Matsunaga M, Matsumoto T, Kang CM, Yamaguchi H, et al (2003) Organization and expression of the Bacillus subtilis sigY operon. J Biochem 134: 935–946.
46. Turner MS, Helmann JD (2000) Mutations in multidrug efflux homologs, sodium isomerases, and antimicrobial biosynthesis genes differentially elevate activity of the sigmaX and sigmaW factors in Bacillus subtilis. J Bacteriol 182: 3210–3219.
47. Wadsophonl I, Bramkamp M (2010) DivIC stabilizes FisO against RasP cleavage. J Bacteriol 192: 5260–5263.
48. Wiegert T, Homuth G, Versteeg S, Schumann W (2001) Alkaline shock induces the Bacillus subtilis sigmaW regulon. Mol Microbiol 41: 59–71.
49. Yoshimura M, Asai K, Sadaie Y, Yoshikawa H (2004) Interaction of Bacillus subtilis SigmaW and its contribution to cell envelope stress responses. Mol Microbiol 67: 830–848.
50. Zweers JC, Wiegert T, van Dijl JM (2009) Stress-responsive systems set specific limits to the overproduction of membrane proteins in Bacillus subtilis. Appl Environ Microbiol 75: 7356–7364.