Mutations upstream of fabI in triclosan resistant Staphylococcus aureus strains are associated with elevated fabI gene expression

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Abstract

Background: The enoyl-acyl carrier protein (ACP) reductase enzyme (FabI) is the target for a series of antimicrobial agents including novel compounds in clinical trial and the biocide triclosan. Mutations in fabI and heterodiploidy for fabI have been shown to confer resistance in S. aureus strains in a previous study. Here we further determined the fabI upstream sequence of a selection of these strains and the gene expression levels in strains with promoter region mutations.

Results: Mutations in the fabI promoter were found in 18% of triclosan resistant clinical isolates, regardless the previously identified molecular mechanism conferring resistance. Although not significant, a higher rate of promoter mutations were found in strains without previously described mechanisms of resistance. Some of the mutations identified in the clinical isolates were also detected in a series of laboratory mutants. Microarray analysis of selected laboratory mutants with fabI promoter region mutations, grown in the absence of triclosan, revealed increased fabI expression in three out of four tested strains. In two of these strains, only few genes other than fabI were upregulated. Consistently with these data, whole genome sequencing of in vitro selected mutants identified only few mutations except the upstream and coding regions of fabI, with the promoter mutation as the most probable cause of fabI overexpression. Importantly the gene expression profiling of clinical isolates containing similar mutations in the fabI promoter also showed, when compared to unrelated non-mutated isolates, a significant up-regulation of fabI.

Conclusions: In conclusion, we have demonstrated the presence of C34T, T109G, and A101C mutations in the fabI promoter region of strains with fabI up-regulation, both in clinical isolates and/or laboratory mutants. These data provide further observations linking mutations upstream fabI with up-regulated expression of the fabI gene.

Keywords: Biocide, Resistance, Triclosan, fabI, Microarray, Cross-resistance, Promoter mutation

Background

Post-genomic research in the past years narrowed down significantly the number of pathway proposed to be suitable as targets for antimicrobial treatment. Based on this, the type II fatty acid biosynthesis pathway shows much promise [1]. One frequently targeted enzyme in this pathway is the enoyl-acyl carrier protein (ACP) reductase enzyme (FabI) as witnessed by the multiple Staphylococcus aureus FabI inhibitors in clinical trial [2-5]. This is further underlined by the fact that FabI is the target of the first line anti-tuberculosis drug isoniazid, diazaborines and the biocide triclosan [1,6]. The latter is a synthetic, non-ionic, chlorinated bis-phenol and is present in many health care products for both hospital and consumer use [7]. It possesses broad spectrum antimicrobial activity against many Gram-positive and Gram-negative bacteria, some fungi [8], and protozoa including Plasmodium falciparum and Toxoplasma gondii [8]. Triclosan, unlike other biocides, at low concentrations has a single intracellular target by binding to the active site of the FabI. It forms a stable
ternary complex with NAD⁺. Triclosan inhibits FabI by al-
losterically blocking the active site, and therefore prevents
bacteria from synthesising fatty acids, which are necessary
for building cell membranes and for division [9].

Several studies have demonstrated that bacteria have
both natural and acquired mechanisms of resistance to
triclosan. Natural resistance is present to varying
degrees in bacterial species, which harbour alternatives
to fabI, (fabK, fabL or fabV) [10-13]. In addition, bio-
degradation has been found to occur in different
environmental species [14]. The primary mechanism of
acquired resistance is due to mutations within the
coding region of fabI, which decrease affinity of the
enzyme to triclosan [9,15-19]. Alternatively, active efflux
of triclosan has been described in several Gram-
negative species, and is mediated by the resistance-
nodulation-division (RND) family of pumps [20]. It has
been shown that triclosan can activate the transcrip-
tional regulator SmeT of the SmeDEF efflux transporter
in Stenotrophomonas maltophilia [21].

In addition to target modification and efflux, also
titration of the target enzymes has been found to confer
resistance. Recent findings have shown the presence of
an additional copy of fabI, horizontally transferred from
S. haemolyticus, in the genome of many S. aureus
isolates with reduced susceptibility to triclosan [22].
Fatty acid biosynthesis is finely regulated in S. aureus
by a feed-forward system that globally controls the expres-
sion of genes involved in this metabolic pathway and
that is dependent on the malonyl-CoA intracellular
levels [23,24]. This metabolite was shown to bind to and
therefore to inhibit the activity of the transcriptional
repressor FapR [23]. FapR is a homodimeric repressor
highly conserved in Gram positive organisms that has
been characterized for its inhibitory function of the
expression of genes involved in the fatty acids and phos-
pholipids biosynthetic pathways [23,24]. Among others,
FapR was shown to directly interact with the promoter of
fabI and to physiologically regulate its expression
[24]. In analogy to the isoniazide resistance conferring
mutation in Mycobacterium tuberculosis [6], increased
amount of the FabI enzyme has been described in triclo-
san resistant S. aureus strains [16], however no gene
expression data are available to sustain this finding.
Furthermore, increased levels of fabI expression have been observed in in vitro adapted S. aureus derivatives, with a possible role of promoter mutations in some of these
strains [25]. Therefore, the aim of this study was to
further characterize mechanisms of resistance in a co-
hort of previously described S. aureus clinical isolates and
in vitro selected mutants with reduced susceptibility
to triclosan by sequencing the putative promoter region
of fabI and by evaluating the levels of gene expression
using microarray analysis.

Results
Promoter sequence analysis
Seven out of thirty-eight (18%) triclosan resistant S. aureus
clinical isolates sequenced were found to have polymor-
phisms in the fabI upstream region (Additional file 1:
Supplementary data S1). Sequence data showed that the
C34T substitution was the most frequent SNP (5 strains),
while the SNPs A101C and T109G were found only once
(Table 1, Figure 1). The latter SNP was found in clinical
isolate QBR-102278-1052 where the insertion of an IS256
element upstream the fabI gene created an eight bp duplica-
tion (AAAAAGTC), which generated the T109G poly-
orphism (Table 1; Figure 1). No mutations were found
in the nineteen triclosan susceptible isolates (Additional
file 1: Supplementary data S1). FabI promoter mutations
were found in 3 out of 9 strains of the group of isolates
without any known triclosan resistance marker, 2/15 in
the strains with sa-fabI mutations and 2/14 in the strains
carrying the sh-fabI gene. When we previously analysed
the sa-fabI locus in the twenty-three in vitro selected triclo-
san mutant strains, we found that all (23/23) had a
mutated sa-fabI gene [22]. Now we found that about
half (13/23) of these had an additional sa-fabI promoter
mutation (Table 1; Figure 1; Additional file 1: Supplementary
data S1). The majority of the mutations in the sa-fabI pro-
moter reflected those found in clinical isolates, except
for the A7G and A72G SNPs and the A101 deletion
(Table 1; Figure 1). Similar to the clinical isolates, the
C34T mutation was the most represented polymorphism
(Table 1; Figure 1). In order to identify other genetic deter-
minants possibly involved in triclosan reduced susceptibil-
ity, seven mutants were further analysed by whole genome
sequencing. These data revealed that only mutations in
the fabI locus were shared by all strains. MO052 was the
only strain analysed by microarray containing additional
SNPs, but no obvious associations between these and
changes in the transcriptome could be made (Table 2 and
Additional file 2: supplementary data S2).

Gene expression analysis
Laboratory mutants
Transcriptomic differences between triclosan susceptible
wild type strains and their resistant isogenic mutants
were analysed by microarrays. Four pairs with different
mutations in the promoter regions were compared
(Table 3 and Table 4). Strains with the following promoter
mutations were analysed: A7G in MO036 and A72G in
MO034 were mutations documented only in laboratory
strains, but not in clinical isolates. C34T in MO035 was
the most frequently observed mutation in both laboratory
strains and clinical isolates. Finally, we also analysed
T109G, the second most frequent mutation in the labora-
atory strains, which was also identified in a clinical isolate
(Table 3, Figure 1). fabI was up-regulated in three of the
Table 1 Genotypes and phenotypes of \textit{in vitro} mutants and clinical isolates with \textit{fabI} promoter mutations

| Strain      | Background | \textit{sa-fabI} promoter** | \textit{sa-fabI}‡ | \textit{sh-fabI}§ | MIC*‡ | MBC*§ | Comment |
|------------|------------|-----------------------------|-------------------|-----------------|-------|-------|---------|
| RN4220     | -          | wt                          | wt                | -               | 1     | 2     |         |
| MW2        | -          | wt                          | wt                | -               | 0.12  | 0.12  |         |
| ATCC6538   | -          | wt                          | wt                | -               | 0.12  | 0.25  |         |
| MO036      | RN4220     | A7G                         | mutated           | -               | 4     | 8     |         |
| MO035      | RN4220     | C34T                         | mutated           | -               | 8     | 8     |         |
| MO047      | RN4220     | C34T                         | mutated           | -               | 4     | 8     |         |
| MO049      | RN4220     | C34T                         | mutated           | -               | 4     | 8     |         |
| MO076      | MW2        | C34T                         | mutated           | -               | 4     | 8     |         |
| CR002      | ATCC6538   | C34T                         | mutated           | -               | 4     | 8     |         |
| MO034      | RN4220     | A72G                         | mutated           | -               | 8     | 8     |         |
| MO077      | MW2        | A101-                        | mutated           | -               | 4     | 32    | 1 bp deletion |
| d7         | ATCC6538   | A101-                        | mutated           | -               | 2     | 8     | 1 bp deletion |
| MO051      | ATCC6538   | T109G                        | mutated           | -               | 4     | 8     |         |
| MO052      | ATCC6538   | T109G                        | mutated           | -               | 8     | 16    |         |
| MO053      | ATCC6538   | T109G                        | mutated           | -               | 4     | 8     |         |
| MO055      | ATCC6538   | T109G                        | mutated           | -               | 4     | 8     |         |
| QBR-102278-1097 | - | C34T                         | mutated           | -               | 0.25  | 32    |         |
| QBR-102278-1889 | - | C34T                         | wt                | -               | 0.5   | 16    |         |
| QBR-102278-1969 | - | C34T                         | wt                | -               | 0.25  | 32    |         |
| QBR-102278-2095 | - | C34T                         | wt                | -               | 0.25  | 32    |         |
| QBR-102278-2546 | - | C34T                         | mutated           | +               | 1     | 64    |         |
| QBR-102278-2363 | - | A101C                        | wt                | +               | 16    | 32    |         |
| QBR-102278-1052 | - | T109G                        | wt                | +               | 0.5   | 64    | IS256 insertion |

*MIC and MBC to triclosan are expressed as mg/L. **Polymorphic sites are indicated counting backwards from the \textit{sa-fabI} start site of \textit{S. aureus} Mu50 (GenBank ID: BA000017; position 1060308). Strains analysed by microarray are indicated in bold. §With the exception of strain QBR-102278-2095, these data have been previously reported \cite{22}. 

Figure 1: Mapping of mutations in the intergenic region upstream \textit{sa-fabI}. The \textit{sa-fabI} upstream region from the \textit{S. aureus} Mu50 genome (GenBank ID: BA000007) is reported. Nucleotides in which mutations have been identified are marked in bold. The positions of the mutations are reported with respect to the first nucleotide preceding the \textit{sa-fabI} start codon and numbered backwards. The nucleotide substitution is described above the mutation position together with the number of clinical isolates (italicised number) and mutant strains carrying that particular mutation. ATCC6538 \textit{sa-fabI} upstream region sequence is identical to Mu50, while the naturally occurring polymorphisms identified in the \textit{wt} strains RN4220 (A29T; GenBank ID: AFGU01000045), ATCC25923 (A213T, A188C; GenBank ID: CP009361), and MW2 (T224A; GenBank ID: BA0000033) with respect to the Mu50 sequence are not reported as they do not affect triclosan susceptibility. The putative $-35$ and $-10$ consensus sequences, identified by BPROM, are underlined. The consensus of the transcriptional repressor FapR recognition sequence is reported as mapped in RegPrecise (underlined) \cite{26} or as previously reported by alignment with the experimentally determined one in the \textit{fapR} upstream region \cite{23,24}. The transcriptional start site (+1TSS) as identified by RNAseq \cite{50} and the ribosomal binding site (SD) are also reported.
| Ref. genome positions* | Annotation | RN4220 derived | ATCC6538 derived | Mu50 derived |
|------------------------|------------|-----------------|------------------|--------------|
| NCTC8325 Mu50          |            | MO034 MO035 MO036 | d2** d7 MO052 | MO079** |
| 120767                 | Capsular polysaccharide synthesis enzyme Cap5B |                  | A 592 T – Met 198 Leu | |
| 208900                 | Pyruvate formate-lyase 1-activating enzyme |                  | G 721 T - Val 241 Phe | |
| 226813                 | Flavohemoprotein |                  | -TA 953 deletion leading to transcription premature truncation | |
| 784247                 | Protein traslocase subunit SecG |                  | T 22 A – Leu 8 Ile | |
| 919922                 | fabI upstream region** |                  | T 109 G | |
| 919929                 | fabI upstream region |                  | A 101 deletion | |
| 919959                 | fabI upstream region |                  | A 72 G | |
| 919997                 | fabI upstream region |                  | C 34 T | |
| 920024                 | fabI upstream region |                  | A 7 G | |
| 920098                 | fabI coding region |                  | G 68 C – Gly 23 Ala | |
| 920331                 | fabI coding region |                  | G 301 T – Asp 101 Tyr | |
| 920469                 | fabI coding region |                  | G 301 T – Asp 101 Tyr | |
| 920641                 | fabI coding region |                  | T 611 G – Phe 204 Cys | |
| 920641                 | fabI coding region |                  | T 611 G – Phe 204 Cys | |
| 1069028                | fabI coding region |                  | T 611 A – Phe 204 Tyr | |
| 1139511                | serine/threonine-protein kinase PrkC |                  | G 1309 A – Val 437 Ile | |
| 1231020                | DNA mismatch repair protein MutL |                  | T 1709 A – Ile 569 Asn | |
| 1238213                | MurD UDP-N-acetylglucosaminyl-L-alanyl-D-glutamate synthetase | | T 338 G – Ile 113 Ser | |
| 1238872                | MurD UDP-N-acetylglucosaminyl-L-alanyl-D-glutamate synthetase | | C 997 G – Leu 333 Val | |
| 1588812                | tRNA methylthiotransferase YqeV |                  | G 824 A – Ile 275 Thr | |
| 2335216                | HTH-type transcriptional regulator SarV |                  | C 140 A – Val 47 Gly | |
| 2774594                | Ica operon transcriptional regulator, IcaR |                  | -TCTTTGCA 417 deletion leading to transcription premature truncation | |

*The nucleotide position refers to the genome of S. aureus NCTC8325 (accession NC_007795) or Mu50 (accession NC_002758). Polymorphic sites of fabI promoter region are indicated counting backwards from the sa-fabI start site of S. aureus Mu50 (GenBank ID: BA000017; position 1060308). **Despite not having fabI promoter mutations the d2 and MO079 mutants have been included in the analysis to check for the presence of mutations (not only in the fabI coding sequence) playing a possible role in triclosan reduced susceptibility.
four tested laboratory strains, with a change in fold expression between 3.47 and 4.97 (Table 3 and Table 4). The \textit{fabI} gene was the highest up-regulated in both MO035 and MO052 (Additional file 2: Supplementary data S2). MO034 was characterized by a higher number of up- and down-regulated genes (Additional file 2: Supplementary data S2) with \textit{fabI} being the third highest regulated probe. In contrast the strain MO036, carrying the A7G mutation, was not found to have an up-regulated \textit{fabI} expression. In this strain, only two genes, not previously described to be involved in the development of triclosan resistance, were slightly up-regulated (Additional file 2: Supplementary data S2). No further significant association were observed between polymorphisms retrieved with the whole genome sequencing and up- and down-regulation of corresponding genes in mutant strains.

**Clinical isolates**

A comparison between triclosan resistant clinical isolates containing mutations in the \textit{fabI} promoter and sensitive prototypical strains was also performed. Four clinical isolates with reduced triclosan susceptibility (Table 3) were compared to the prototypical, triclosan susceptible \textit{S. aureus} strain ATCC25923 (MIC and MBC of 0.06 and 1 mg/L respectively). Two of these strains (QBR-102278-1889 and QBR-102278-1969) had the same \textit{fabI} promoter

### Table 3 Overview of the changes in gene expression in laboratory mutants and clinical isolates

| Comparison strains | Promoter mutation | Up-regulated genes | Down-regulated genes | Fold increase \textit{fabI} expression |
|--------------------|-------------------|--------------------|----------------------|--------------------------------------|
| MO034 vs RN4220    | A72G              | 154                | 125                  | 4.97                                 |
| MO035 vs RN4220    | C34T              | 7                  | -                    | 3.92                                 |
| MO036 vs RN4220    | A7G               | 2                  | -                    | -                                    |
| MO052 vs ATCC6538  | T109G             | 3                  | 20                   | 3.47                                 |
| QBR-102278-1889 vs ATCC25923 | C34T | 76 | 18 | 4.86 |
| QBR-102278-1969 vs ATCC25923 | C34T | 78 | 36 | 6.44 |
| QBR-102278-2363 vs ATCC25923 | A101C | 122 | 28 | 4.26 |
| QBR-102278-1052 vs ATCC25923 | T109G | 198 | 8 | 47.6 |

### Table 4 \textit{fabI}/\textit{gyrA} ratio of laboratory and clinical strains

| Strains                          | \textit{Log}_2 \textit{fabI}/\textit{gyrA} ratio* | \textit{t} test vs parental strain | \textit{t} test vs ATCC 6538 | \textit{t} test vs ATCC25923 |
|----------------------------------|---------------------------------------------------|-----------------------------------|-----------------------------|----------------------------|
| Laboratory strains               |                                                   |                                   |                             |                           |
| RN4220 (n = 6)                   | 1.69                                              |                                   |                             |                           |
| MO034 (n = 5)                    | 2.49                                              | 0.04                              |                             |                           |
| MO035 (n = 5)                    | 3.37                                              | 0.0002                            |                             |                           |
| MO036 (n = 3)                    | 1.41                                              | ns                                |                             |                           |
| MU50 (n = 5)                     | -0.28                                             |                                   |                             |                           |
| ATCC6538 (n = 4)                 | 0.14                                              |                                   |                             |                           |
| MO052 (n = 4)                    | 2.14                                              | 0.0002                            |                             |                           |
| ATCC25923 (n = 4)                | -0.51                                             |                                   |                             |                           |
| Clinical strains                 |                                                   |                                   |                             |                           |
| QBR-102278-1052 (n = 4)          | 4.22                                              | <0.0001                           | <0.0001                     |                           |
| QBR-102278-1889 (n = 4)          | 1.25                                              | 0.0026                            | 0.0137                      |                           |
| QBR-102278-1969 (n = 4)          | 1.56                                              | 0.0002                            | 0.0059                      |                           |
| QBR-102278-2363 (n = 4)          | 1.16                                              | 0.004                             | 0.016                       |                           |
| QBR-102278-1016 (n = 3)          | 0.23                                              | ns                                | ns                          |                           |
| QBR-102278-1027 (n = 4)          | 0.13                                              | ns                                | ns                          |                           |
| QBR-102278-2628 (n = 5)          | 0.03                                              | ns                                | ns                          |                           |

*\textit{Log}_2 ratio was independently determined for each chip using the data before stage-wise quantile normalization, by subtracting the \textit{Log}_2 mean \textit{gyrA} values obtained from the eight probes for \textit{gyrA} printed on the chip from the \textit{Log}_2 mean \textit{fabI} values from the two probes. Ratio was then further averaged for the number of chips used by strains (n). Student’s unpaired \textit{t}-test was performed by comparing the mutants to their parental strains in the case of laboratory strains, or to two reference strains in the case of clinical isolates. ns = not significant.
mutation (C34T) as the laboratory strain MO035. One strain (QBR-102278-1052) had the mutation T109G, also found in MO032. Finally, one strain (QBR-102278-2363) showed a mutation (A101C) in a region deleted in two laboratory strains, which were however not tested by microarrays (Table 3, Figure 1). A higher number of up- or down-regulated genes were found in these clinical isolates than in laboratory strains, except MO034. This was expected, since the comparison could not be made between isogenic strains. Furthermore, the genetic diversity of these strains may be augmented by the presence of plasmids not present in ATCC25923. Nevertheless, in all clinical strains, fabI was up-regulated, from 4.26 to 47.6 fold (Table 3, Table 4, and Additional file 2: Supplementary data S2). Also the Log$_{_{10}}$ fabI/gyrA ratios were found to be significantly higher in the four clinical strains with decreased sensitivity to triclosan when compared to the two reference strains ATCC6538 and ATCC25923 (Table 4). This was in contrast to 3 triclosan susceptible strains (QBR-102278-1016, QBR-102278-1027, and QBR-102278-2628; described in Additional file 1: Supplementary data S1), which showed ratios comparable to the reference strains (Table 4). Quantitative real-time PCR confirmed a statistically significant increase of comparable entity in the expression of the fabI gene of clinical isolates when compared to reference strains (using gyrA as housekeeping control). The increase was for the fabI gene of QBR-102278-1052, QBR-102278-1889, QBR-102278-1969, and QBR-102278-2363 respectively 36.9, 9.0, 15.6, and 7.7 fold compared to ATCC6538 and of 9.9, 2.4, 4.2, and 2.1 fold when compared to ATCC25923. QBR-102278-1052, the strain with the highest up-regulation of fabI and the highest MBC was also characterized by the presence of an IS256 element upstream of the promoter. Other possible mechanisms of triclosan resistance in these clinical strains were evaluated by identifying the genes commonly up- or down-regulated in the four clinical isolates. 37 genes were found to be up-regulated in all 4 strains (Tables 5, 6 and Additional file 3: Supplementary data S3). Furthermore, 6 genes were down-regulated. Apart from fabI, all genes found to be up- or down-regulated were of chromosomal origin, meaning that, at least in the four clinical isolates tested in the present study, triclosan resistance is not plasmid-mediated at the level of gene expression. Gene ontology (GO) annotations were available for 29 genes of all 43 differently regulated genes (Additional file 3: Supplementary data S3). Genes involved in transport (8 genes) or in membrane structure/metabolism (12 genes) were the most represented. These intrinsic differences in bacterial membrane properties could influence triclosan tolerance independently from fabI, for example by altering triclosan trafficking through the membrane. However, we did not find genes coding for efflux pumps or known antibiotic/biocide resistance genes to be up-regulated (Tables 5 and 6).

Discussion

Several mechanisms inducing reduced triclosan susceptibility have been described in both Gram-positive and Gram-negative organisms [9-22]. Amongst them, mutations in the coding regions of the fabI gene or an increase in its expression level have been related to triclosan resistance [9,15-19].

The analysis of the fabI upstream region in triclosan resistant clinical isolates and in vitro selected mutant strains revealed the presence of nucleotide changes with respect to triclosan susceptible strains. Interestingly three mutations (T109G, A101C, and A101-del) were found to occur within the FapR DNA recognition sequence. These mutations are likely to interfere with FapR binding, therefore reducing its inhibitory effect on fabI transcription. More importantly, the thymine in the 109 position was found to be highly conserved among the FapR DNA recognition sequences [26], while the 101 adenine was previously showed to be specifically recognized and bound by the Arg56B residue of FapR [23]. It is noteworthy that the A72G mutation, present only in a laboratory mutant strain, was found to occur between the predicted −35 and −10 promoter sequences. On the other hand no clear indication on the mode of action of the C34T mutation, located 7 bp downstream the transcription start site, could be found. The high frequency of this mutation in clinical isolates points to an important regulatory effect, which might be hypothesised to be linked to post-transcriptional regulation. A hypothesis strengthened by the high level of sequence identity, including a complete match of the sequence preceding C34, between the staphylococcal fabI 5′-prime UTR to Enterococcus faecalis [27]. However further studies will be required to determine the exact nature of such regulatory events.

Our data indicate that mutations in the fabI upstream region were not always found to be associated to other previously described triclosan resistance mechanisms. Indeed, fabI promoter mutated strains were found either alone or associated to the presence of the sh-fabI gene or mutations in the sa-fabI. In particular we found three triclosan resistant clinical strains with mutations in the fabI promoter, but not in the coding region. In two of these strains analysed by microarray an up-regulation of the fabI gene was also observed suggesting that these promoter mutations in fabI may induce overexpression and participate in triclosan resistance. Although having mutation in the promoter region, the strain MO036 didn’t display higher level of fabI gene expression. The most plausible cause of triclosan resistance in this strain is the observed mutation in the coding region of the gene. However, isaA, overexpressed in MO036, could also be an additional strategy contributing to triclosan resistance, since its involvement has been recently mentioned for fusidic acid resistance, by altering cell wall metabolism and therefore cell properties [28].
| Genes                                      | QBR-102778 |
|--------------------------------------------|------------|
|                                            | -1889      | -1969      | -2363      | -1052      |
| cycA / putative D-serine/D-alanine/glycine transporter | 2.5        | 3.2        | 4.7        | 6.5        |
| ditD / putative lipoteichoic acid biosynthesis protein | 2.9        | 2.3        | 2.3        | 8.9        |
| fabI / enoyl-acyl-carrier-protein reductase (NADH) | 4.8        | 6.4        | 4.3        | 47.6       |
| fruA / PTS transport system, fructose-specific IIABCcomponent | 5.3        | 4.9        | 11.1       | 11.1       |
| gyrA / DNA gyrase subunit A | 2.6        | 2.4        | 2.2        | 3.4        |
| hlb / phage protein | 3.2        | 2.6        | 4.9        | 10.5       |
| isaB / hypothetical protein | 2.8        | 2.4        | 2.5        | 5.4        |
| mprF / putative membrane protein | 4.5        | 2.2        | 6.8        | 8.3        |
| murA1 / putative UDP-N-acetylglucosamine1-carboxyvinyltransferase | 4.6        | 4.3        | 8.3        | 17.1       |
| rho / transcription termination factor | 2.2        | 2.2        | 3.3        | 7.4        |
| rir1 / ribonucleoside-diphosphate reductase alphachain | 2.4        | 2.1        | 2.4        | 4.0        |
| SATW20_19350 / phage protein | 6.0        | 5.9        | 10.6       | 17.9       |
| SAV0240 / SATW20_02420 / flavohemoprotein | 10.6       | 18.4       | 26.0       | 13.7       |
| SAV0348 / SATW20_04160 / hypothetical protein | 2.1        | 2.1        | 3.5        | 2.3        |
| SAV0465 / SATW20_05330 / putative exported protein | 7.0        | 3.8        | 7.7        | 13.7       |
| SAV0663 / SATW20_07380 / conserved hypothetical protein | 4.8        | 5.4        | 9.0        | 14.1       |
| SAV0699 / SATW20_07740 / putative phosphofructokinase | 2.5        | 2.6        | 4.7        | 3.9        |
| SAV0944 / SATW20_09440 / thioesterase superfamily protein | 3.6        | 2.6        | 8.9        | 2.0        |
| SAV1356 / SATW20_13570 / sodiumalanine symporter family protein | 2.1        | 2.3        | 2.9        | 4.3        |
| SAV1573 / SATW20_15690 / putative exported protein | 3.0        | 2.2        | 4.6        | 5.5        |
| SAV1853 / SATW20_18470 / putative membrane protein | 2.7        | 2.5        | 3.0        | 2.9        |
| SAV1914 / SATW20_19090 / putative oxygenase | 3.9        | 2.7        | 6.6        | 6.6        |
| SAV1947 / hypothetical protein | 22.4       | 6.3        | 28.3       | 9.3        |
| SAV2032 / SATW20_20150 / membrane anchored protein | 3.3        | 2.2        | 3.3        | 3.7        |
| SAV2184 / SATW20_23220 / putative membrane protein | 4.8        | 4.1        | 7.2        | 6.0        |
| SAV2253 / SATW20_23870 / xanthine/uracil permease family protein | 6.2        | 3.4        | 3.8        | 3.7        |
| SAV2253 / SATW20_23870 / xanthine/uracil permeases family protein | 4.3        | 2.2        | 2.4        | 3.0        |
| SAV2335 / SATW20_24670 / putative membrane protein | 2.5        | 2.3        | 3.8        | 2.6        |
| SAV2368 / SATW20_25000 / hypothetical protein | 5.1        | 3.2        | 6.6        | 6.7        |
| SAV2368 / SATW20_25000 / putative lipoprotein | 9.8        | 6.6        | 16.1       | 10.0       |
| SAV2383 / SATW20_25130 / putative exported protein | 3.0        | 3.3        | 4.0        | 7.4        |
| SAV2403 / SATW20_25330 / putative nitrite transporter | 3.5        | 2.4        | 2.8        | 6.1        |
| SAV2404 / SATW20_25340 / putative membrane protein | 6.7        | 4.9        | 6.2        | 3.3        |
| SAV2412 / SATW20_25420 / ABC transporter ATP-binding protein | 10.1       | 8.5        | 16.3       | 25.5       |
| SAV2413 / SATW20_25430 / ABC transporter permease | 6.2        | 4.9        | 9.6        | 18.4       |
| SAV2413 / SATW20_25430 / transport system membrane protein | 3.9        | 3.4        | 4.7        | 8.0        |
| SAV2414 / SATW20_25440 / extracellular solute-binding lipoprotein | 2.4        | 2.2        | 3.1        | 5.0        |
| scn / staphylococcal complement inhibitor SCIN | 64.7       | 63.5       | 47.7       | 85.1       |
| trap / signal transduction protein TRAP | 10.6       | 9.5        | 16.7       | 21.7       |
| xpt / putative xanthine phosphoribosyltransferase | 7.8        | 6.5        | 12.5       | 12.9       |
Previous experiments showed that mutations in the coding region of fabI varied between mutant strains and clinical isolates [22]. In contrast, there was good overlap between in vitro selected fabI promoter mutations and those of clinical strains in the present study (Figure 1). On the other hand, phenotypic differences were evident between mutants and clinical isolates carrying fabI promoter mutations (Table 1). Indeed mutant strains showed higher MICs values and lower MBCs values with respect to clinical isolates. The higher MICs could be explained by the technical constrain imposed by the mutant selection method [22] in which the active multiplying cells need to be grown in presence of triclosan concentrations sufficient to isolate resistant mutants from wt strains. On the other hand in vivo concentrations of triclosan could be lower, potentially transient and acting on bacteria with greater generation times. These three aspects, not reproducible in in vitro conditions, could have allowed for a different selection of triclosan resistant mutants in the natural environment, including in the human host. Such phenotypic differences were also reflected by the absence of in vitro generated mutants showing the sole fabI upstream mutation (Table 1) confirming that our laboratory mutant selection strategy couldn’t select triclosan resistant strains without mutations in the fabI coding region, while clinical isolates could evade triclosan bactericidal activity by increasing the baseline expression of fabI through the sole promoter mutations. However these divergences do not indicate any reduction of fitness neither in the lab strains nor the clinical isolates [29].

The technical constrain for selecting high MIC mutants in vitro is due to the drug concentration used in the screening of active multiplying cells. Probably in vivo concentrations drug concentrations can be lower and potentially transient and the bacterial generation time is greater. These three aspects could allow a different selection of mutants in vivo. Considering lower and transient drug concentration and possibly greater generation time, it is possible that an increased baseline expression may allow for out-titration of killing effect.

Microarray analysis confirmed that in all clinical strains and the majority of lab mutants, promoter mutations were associated with an up-regulation of fabI transcription (Tables 4, 5 and 6). Increased expression of fabI gene has been described in triclosan-resistant S. aureus clinical isolates [16], S. epidermidis mutants [30], laboratory mutants of S. aureus [25], E. coli and Salmonella [31-33]. Promoter mutation has never been linked to fabI over-expression despite mutation in the fabI upstream region were previously identified in adapted S. aureus USA300 isolates [25]. Interestingly, the exposure of S. aureus [34], Salmonella enterica or E. coli [35] to triclosan does not necessarily lead to fabI up-regulation. One of our clinical strains (strain QBR-102278-1052), distinguished itself by a very high level (approx. 50 fold up-regulation) of fabI expression. It is of notice that quantitative real-time PCR confirmed the particularly high fabI expression of QBR-102278-1052 isolate. In this clinical strain, the insertion of an additional sh-fabI allele was also documented. Still, the observed increase in signal on the microarray was exclusively due to sa-fabI hybridization, since the two oligonucleotide probes printed on the array were able to discriminate sa-fabI from sh-fabI. Rather, we found an IS256 insertion sequence element 114 bp upstream of and in the same direction as the fabI gene in this strain. Multiple copies of IS256, not associated with any resistance genes, have been found in the chromosome of S. aureus [36], but also in many strains of Enterococcus faecalis and E. faecium [37]. The formation of a potent hybrid promoter containing IS256 could be a new additional mechanism leading to high level of fabI expression and decreased susceptibility to triclosan, which however remains to be proven experimentally. This hypothesis would be supported by the observation of a high level resistance to mexiticillin and fluoroquinolones in S. aureus induced by the insertion of IS256 upstream of the lltm and norA genes respectively [38,39]. A similar mechanism was also described in Staphylococcus sciuri, in which mexiticillin resistance was linked to the insertion of IS256 upstream of the gene coding for a mecA homolog [40].

Our microarray analysis of triclosan-resistant clinical isolates did not reveal any efflux-mediated resistance mechanism, neither chromosomally-encoded, nor plasmid-mediated. This is in agreement with previous studies showing that

### Table 6 Genes down-regulated in triclosan resistant S. aureus clinical isolates with respect to ATCC25923

| Genes | QBR-102778 -1889 | QBR-102778 -1969 | QBR-102778 -2363 | QBR-102778 -1052 |
|-------|----------------|----------------|----------------|----------------|
| fda / fructose-bisphosphate aldolase class I | 0.03 | 0.02 | 0.03 | 0.04 |
| SATW20_01020 / putative hydratase | 0.17 | 0.15 | 0.29 | 0.30 |
| SATW20_28340 / putative N-acetyltransferase | 0.11 | 0.07 | 0.06 | 0.03 |
| SAV0801 / hypothetical protein | 0.17 | 0.16 | 0.15 | 0.13 |
| SAV2515 / SATW20_26360 / transmembrane protein smpB | 0.38 | 0.46 | 0.24 | 0.38 |
| SAV2643 / SATW20_27810 / putative membrane protein | 0.45 | 0.38 | 0.35 | 0.31 |
there was no significant increase in the triclosan MBC for *S. aureus* strains carrying plasmid-borne *qac* genes coding for multidrug efflux pumps [41]. Similarly, over-expression of the chromosomal *norA* multidrug transporter gene did not lead to triclosan resistance [42]. Efflux-mediated resistance to triclosan is however not to be excluded in Gram-positive bacteria. Indeed, it has been recently shown that, out of 21 *S. haemolyticus* clinical strains, an inhibition of efflux pump activity by carbonyl cyanide-m-chlorophenylhydrazone (CCCP) significantly decreased triclosan MIC in four strains [43] suggesting the possible presence of a still unidentified efflux system with triclosan as a substrate. Despite this fact, efflux-mediated triclosan resistance to date still remains restricted to Gram-negative species [20,21]. Other mechanisms inducing an alteration of membrane metabolism, structure or trafficking in *S. aureus* could not be excluded. This hypothesis would be supported by the identification of several up-regulated genes involved in such mechanisms in clinical strains or *isaA* up-regulation in the laboratory mutant MO036.

Apart from the *fabI* gene or efflux pumps, evidence for other mechanisms of resistance to triclosan is scarce. Prolonged exposure of MRSA to triclosan-impregnated silicon elastomer resulted in the selection of small colony variants resistant to triclosan, but the underlying mechanism is still not understood and may be non-specific, involving a reduction in energy generation and/or transport and the down-regulation of functions such as cell wall synthesis [44]. High-throughput methods (proteomics, genomics) have been recently applied to laboratory strains of *S. typhimurium* and *E. coli* grown in absence of triclosan in order to reveal inherent mechanisms of resistance to triclosan based on changes in genes or proteins expression [32,33,45]. Apart from the consistent up-regulation of *fabI*, relatively few new mechanisms were proposed. It has been postulated that the increased expression of dehydrogenases and oxidoreductases using NAD+ as a co-factor, could bind and capture triclosan, reducing its effective intracellular concentration [32,45]. In the present study, apart from *fabI*, only one putative flavohemoprotein (SATW20_02420) containing identified binding sites for NAD or FAD was highly up-regulated in the clinical strains. In most cases no complete operon was found to be over-expressed in the triclosan resistant clinical isolates. Exceptions are the co-transcribed *fruA* and *fruB* genes and the three genes for an amino acid ABC uptake system (SAV2412-4). This is in accordance with previous data, which had shown that exposure of *S. aureus* to triclosan leads to the de-regulation of branched amino acid uptake and carbohydrate metabolism including changes in expression of *fruA*, *fruB* and *xprT* [34]. Albeit the partial overlap of data between our characterisation of resistant isolates and the work on triclosan toxicity, no clear metabolic correlation can be drawn, which links triclosan and fatty acid metabolism to the observed changes in gene expression. In that respect, the current analyses of the six clinical isolates devoid of any resistance markers could also provide further insights, especially if *fabI* turns out not to be overexpressed in these strains.

To our knowledge, this is the first extensive microarray analysis comparing both *in vitro* generated mutants and clinical isolates of *S. aureus* resistant to triclosan. The comparison of the mutants and their parental strains enables us to link genetic variations to phenotypic changes more directly. For clinical strains, comparisons between resistant strains and a prototypical strain do not allow such direct conclusion and also relate to the choice of the prototypical strain. These results were therefore confirmed by determining *fabI*/gyrA ratio. To definitely challenge the hypothesis that mutations in the promoter of *fabI* lead to overexpression of the gene and consequently to a reduced susceptibility, prospective genetic manipulation would have been a more direct approach. Nevertheless, whole genome sequencing revealed in three out of four laboratory mutant tested by microarray, that the only mutation susceptible to explain change in *fabI* expression was located in *fabI* promoter region. Furthermore, the fact that the same *fabI* promoter mutations were found in all clinical strains with overexpression of their *fabI* gene, indirectly provide the evidence for the involvement of such mutations in *fabI* up-regulation and possibly in triclosan reduced susceptibility.

**Conclusion**

In conclusion, molecular changes in the promoter region of *fabI* were identified together with *fabI* over-expression and triclosan resistance. As such this overexpression of *fabI* has the potential to determine cross-resistance to novel compounds in clinical trial [2-5]. This adds to the recently described mutations within *fabI* selected by the novel compound AFN-1252 which confer cross-resistance to triclosan [46]. It should be noted that in *S. aureus fabI* up-regulation acts almost always in addition to the other triggers of triclosan resistance, such as mutations in coding regions of the *fabI* gene or the insertion of an addition allele derived from *S. haemolyticus*. Importantly, we could not link triclosan resistance in staphylococci to the presence or over-expression of efflux systems, either of plasmid or chromosomal origin, a mechanism known to contribute to resistance in Gram-negative organisms [47] or to the resistance to any other antimicrobial drug [48]. In addition we have solid evidence of the *fabI* up-regulation in four triclosan resistant clinical isolates for which the intrinsic genetic diversity between clinical isolates so far had eluded comparison of gene expression. Indirect evidences support the relationship between *fabI*
overexpression and the presence of mutations mapped in the FabI promoter region. This hypothesis is further strengthened by the finding of the same mutations in fabI-overexpressing laboratory mutants, which, most importantly, were shown to not carry any other mutation in their genome that could be associated to triclosan resistance. This combined data in laboratory mutants and clinical isolates opens new avenues to explore mechanisms of triclosan resistance in S. aureus.

Methods

Bacterial strains

Sixty-five S. aureus strains with reduced susceptibility to triclosan were previously selected from a collection of 1602 clinical isolates by performing standard MIC and MBC assays [22,48,49]. Of these, fifteen strains with mutations in the fabI coding sequence, fourteen with an additional chromosomal sh-fabI allele (from S. haemolyticus), and nine without any known triclosan “resistance” marker were investigated in this work for fabI-promoter mutations (Additional file 1: Supplementary data S1). As a control nineteen triclosan susceptible isolates were included in the analyses (Additional file 1: Supplementary data S1). In addition twenty-three independent mutants (Additional file 1: Supplementary data S1), with reduced susceptibility to triclosan, were also analysed. These strains have been previously selected by single-exposure of ATCC6538, MW2, and Mu50 reference strains to 0.5 mg/L of triclosan in solid medium or by multiple step-growth on liquid medium with increasing triclosan concentrations (from 0.25 mg/L to 4 mg/L) as in the case of the RN4220 laboratory strain [22].

No ethical approval was required to obtain the isolates used in the study. No ethical approval was required to use the clinical isolates in this study. Isolates were obtained during routine microbiological investigations and were not part of a clinical trial.

Molecular analysis

The upstream region of the fabI gene was amplified in the fifty-seven clinical isolates and in the twenty-three laboratory mutants. DNA was amplified using standard PCR conditions and the following primers: 5’-ATCA TCTTCGTGCGTATTATC-3’ and 5’-TTCAGCTCT TTACGGCTA-3’ (Eurofins MWG Operon, Germany). PCR products were sequenced by the Sanger method (Eurofins MWG Operon, Ebersberg, Germany). A selection of S. aureus fabI upstream sequences has been deposited in GenBank (accession nos. KF583951- KF583970). The putative −35 and −10 sequences have been predicted using the BProM tool (http://www.softberry.com/berry.phtml), while the FapR recognition sequence was mapped by mean of the data available on the RegPrecise database [26]. The transcriptional start site was identified by direct visualisation of RNA-seq alignment data retrieved from the NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra/). More precisely the illumina HiSeq data were previously generated by sequencing of the RNA extracted from a S. aureus Newman strain at the early log phase (SRA Experiment ID: DRX011556, SRA Sample ID: DR5011392) [50]. Whole-genome sequencing was performed by the Institute of Applied Genomics (University of Udine, Italy) using an Illumina Genome Analyzer II platform (Illumina, San Diego, California, USA) for the in vitro selected mutants d2, d7, MO034, MO035, MO036, MO052, MO079 and their isogenic wild type strain RN4220, ATCC6538, and Mu50 (Table 2). Mutants to be sequenced have been selected on the basis of their fabI promoter sequence in order to analyse one strain for each one of the previously identified mutations. Strains d2 and MO079 have been also included in order to check for genetic changes, other than sa-fabI mutations, possibly related to their triclosan reduced susceptibility phenotypes. Sequences of both wt strains and mutant strains, were aligned to the reference genome of S. aureus NCTC8325 (accession NC_007795), except for Mu50 and MO079 that were aligned to the Mu50 genome (accession NC_002758), using the Mosaic Assembler suite (The MarthLab, Boston College, Massachusetts, USA). Single nucleotide polymorphisms (SNPs), insertions and deletions (INDELS) were retrieved with VarScan software [51]. SNPs and INDELS of the wt strains obtained from the alignment with the GenBank reference genome were subtracted from those found by aligning the mutant strain with the reference.

Statistical analysis

Fisher’s exact test was applied to assess if the differences in the number of clinical isolates with fabI promoter mutations among the three groups of strains defined by known triclosan resistance marker were statistically significant.

Gene expression analysis

Array design and production

Probe design was performed by the CustomArray Design Service. Probes of 35–40 bp length were selected based on melting temperature (Tm), complexity, secondary structure, GC-content, and specificity. A total of 12 000 capture probes were finally used. Furthermore, the array also contained quality control spots, non-specific probes derived from phages, plants, virus and bacteria, as well as empty, oligonucleotide-free spots. The entire genomes of Mu50 and TW20 were covered in the microarrays, plus additional elements as listed in the Additional file 4: Supplementary data S4. Arrays were synthesized on a
strains were grown overnight in 10 ml tryptic soy broth (TSB) at 37°C at 80 rpm. The cultures were diluted 1:100 in pre-warmed TSB and grown to logarithmic phase (OD_{570} = 0.6). 2 ml of each culture (1–5 × 10^8 colony forming units) was harvested in 4 ml of RNAProtect reagent (Qiagen), incubated for 5 min at room temperature and centrifuged for 10 min at 5000 × g. The pellet was then processed directly for RNA extraction or stored at −80°C for later processing.

**Bacterial growth**

*S. aureus* strains were grown overnight in 10 ml tryptic soy broth at 37°C at 80 rpm. The cultures were diluted 1:100 in pre-warmed TSB and grown to logarithmic phase (OD_{570} = 0.6). 2 ml of each culture (1–5 × 10^8 colony forming units) was harvested in 4 ml of RNAProtect reagent (Qiagen), incubated for 5 min at room temperature and centrifuged for 10 min at 5000 × g. The pellet was then processed directly for RNA extraction or stored at −80°C for later processing.

**RNA purification**

Total RNA was extracted using RNeasy Mini Kit (Qiagen), according to the manufacturer’s instructions, using 50 U recombinant lysostaphin (Sigma) followed by incubation for 5 minutes with 1 ml of hot Qiazol (Qiagen) to lyse bacteria. Bacteria were further disrupted by vibration with 50 mg of acid-washed glass beads (Sigma) using a Mickle Vibratory Tissue Disintegrator (Mickle Laboratory Engineering) at maximum speed. Contaminating DNA was removed using DNA-free™ Kit (Applied Biosystems) and RNA quality tested on an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA concentration and purity were determined by Nanodrop® ND-1000 spectrophotometer (Thermo Scientific). For each strain, at least 4 RNA samples were prepared from independent cultures.

**RNA labelling and fragmentation**

Isolated, unamplified RNA was labelled with Cy5, using ULS™ Labeling Kit for CombiMatrix arrays (Kreatech Biotechnology), according to the manufacturer’s instructions. RNA was finally fragmented with RNA Fragmentation Reagents (Ambion™).

**Array hybridization**

12 K Customarrays were hybridized with 2 μg of labelled, fragmented RNA, according to information provided by the manufacturer (Customarray/CombiMatrix Incorporated). Microarrays were scanned using the Packard ScanArray4000 array scanner and software (ScanArray, version 3.1, Packard BioChip Technologies). All arrays were scanned with incremental laser power from 15 to 100%. Data were extracted with Microarray Imager software (version 5.8.0, Combi Matrix) and spot intensity expressed as median intensity.

**Data analysis**

Scanning data with similar median fluorescence intensity were chosen for analysis. Fluorescence values of spots with maximal intensity (signal saturation) at the chosen laser intensity were extrapolated by linear regression, using values gathered from the two next lower laser intensities.

**Gene filtering**

Non-specific binding was determined from fluorescence values of all non-specific probes. The cut-off for specific binding was set as the upper 95% confidence interval of the mean signal intensity of the non-specific probes. For each comparison, probes were excluded when the mean values for both strains to be compared were under the determined cut-off. For comparisons involving *in vitro* generated mutants derived from plasmid-free strains RN4220 or ATCC6538, analysis was performed using only probes sets gathered from the genomes of *S. aureus* TW20 and Mu50.

**Data transformation, normalization and analysis**

The fluorescence values were log2 transformed. For each set of comparison, stage-wise quantile normalization was performed, using a script written in the statistical computing environment of R (R Development Core Team, 2011), according to Deshmukh et al. [52]. Significantly differentially regulated genes were determined by using the Significance Analysis of Microarrays method (SAM, Excel Add-in version 4.0) originally developed at Stanford University lab [53]. For each comparison, the delta value was set to obtain a conservative median false discovery rate (FDR) of 1% and the fold change cut-off value was set to 2. For investigating common up- or down-regulated genes in the 4 triclosan resistant clinical strains, the FDR value was set to 5%.

**Quantitative real-time PCR**

Reverse transcription of total RNA to single-stranded cDNA was performed on selected laboratory strains and clinical isolates using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). Quantitative real-time PCR was carried out using SYBR® Green PCR Master Mix (Applied Biosystems) and the reactions were performed in triplicate, according to the manufacturer’s instructions, using a 7500 Fast Real-time PCR System (Applied Biosystems). The *fabl* gene was amplified using the primers 5′-GT CCAATCCGTACATTAAGTGCA-3′ and 5′-TCACCTG TAACGCCACTTGATAA-3′. The results were normalised to the housekeeping gene *gyrA* amplified using the primers 5′-ACGTCAACGTATTGTTGTCACTG-3′ and 5′-TTAGCACATCAATAACGACAGC-3′. Transcription levels were determined using the 2^ΔΔCT method [54].

**Availability of supporting data**

The data sets supporting the results of this article are available in the ArrayExpress repository, (http://www.ebi.ac.uk/arrayexpress/) under accession numbers A-MEXP-
2362 (S. aureus array design) and E-MTAB-2127 (microarray raw results).

Additional files

Additional file 1: Supplementary data S1: Relevant information of S. aureus strains analysed.

Additional file 2: Supplementary data S2: Statistically significant genes (up- or down-regulated). FabI-specific probes are highlighted in yellow. In bold, the mean value of the fold increase/decrease. Gene are presented in the decreasing fold change for the upregulated gene and in increase fold change for the downregulated genes. The fold change values of every single probe are also indicated. Probes are described as follow: Probe ID/Probe sequence/Gene Name/NCBI Protein Reference Sequence number/Protein product name.

Additional file 3: Supplementary data S3: detailed informations on the genes commonly up- or down-regulated in the 4 clinical S. aureus characterized by a reduced susceptibility to triclosan. Described are the gene locus and UniProt ID as found for the TW20 or MUS0 genomes, as well as the associated KEGG orthology and gene ontologies (GO) when available. GO terms were organized in 3 categories: “Biological Process”, “Molecular Function” and “Cellular Component”.

Additional file 4: Supplementary data S4: list of genome sequences and respective accession numbers used for probes design.

Abbreviations

Acp: Enoyl-acyl carrier protein; NAD: Nicotinamide adenine dinucleotide; RN: Resistance-nodulation-division; SNP: Single nucleotide polymorphism; IS256: Insertion sequence element 256; MIC: Minimal inhibitory concentration; MBC: Minimal bactericidal concentration; GO: Gene ontology; CCCP: Carbonyl cyanide-m-chlorophenylhydrazone; MRSA: Methicillin-resistant Staphylococcus aureus; TSB: Tryptic soy broth; INDELs: Insertions and deletions.

Competing interests

The authors declare no financial and no non-financial conflict of interest. IM and DRK were employees of Quotient Bioresearch at the time of the study.

Authors’ contributions

DG: participated in the experimental design, participated in the custom array design, performed and analysed microarray experiments, wrote in part the manuscript. LF: constructed and characterised in vitro mutants, performed genome analysis, wrote in part the manuscript. MLC: constructed and characterised in vitro mutants. LB: constructed in vitro mutants. DK: performed phenotypic testing of clinical isolates. IM: performed phenotypic testing of clinical isolates and discussion of results. CRL: participated in chip design and performance of microarray analysis. SLL: participated in the experimental design and manuscript writing. MRO: participated in project setup and experimental design, wrote in part the manuscript. All authors read and approved the final manuscript.

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