Regulation of Energy Metabolism by the Extracytoplasmic Function (ECF) σ Factors of Arcobacter butzleri

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Abstract

The extracytoplasmic function (ECF) σ factors are fundamental for bacterial adaptation to distinct environments and for survival under different stress conditions. The emerging pathogen Arcobacter butzleri possesses seven putative pairs of σ/anti-σ factors belonging to the ECF family. Here, we report the identification of the genes regulated by five out of the seven A. butzleri ECF σ factors. Three of the ECF σ factors play an apparent role in transport, energy generation and the maintenance of redox balance. Several genes like the nap, sox and tct genes are regulated by more than one ECF σ factor, indicating that the A. butzleri ECF σ factors form a network of overlapping regulons. In contrast to other eubacteria, these A. butzleri ECF regulons appear to primarily regulate responses to changing environments in order to meet metabolic needs instead of an obvious role in stress adaptation.

Introduction

Arcobacter spp. are Gram-negative, small, motile and spiral-shaped bacteria belonging to the Campylobacteraceae family. Arcobacter is currently comprised of fifteen species [1,2]. Within this genus, A. butzleri, A. cryaerophilus and A. skirrowii are associated with animal and human diseases, such as reproductive disorders, mastitis and diarrhoea in animals [3–6], and enteritis and occasionally bacteraemia in humans [7–10]. Arcobacter spp. have been classified in 2002 as emerging pathogens by the ICMSF (International Commission on Microbiological Specifications for Foods) [11]. The main reservoirs for A. butzleri are water environments, especially sewage and coastal waters [12–14]. In 2007 the complete genome of the human clinical isolate A. butzleri RM4018 was sequenced [15]. The bacterium appears to have a large number of signal transduction systems, indicating that it associates with RNA polymerase and mediates transcriptional activation of its target genes [19]. The ECF anti-σ factor is released from the membrane, where it associates with RNA polymerase and mediates transcriptional activation of its target genes [19]. The ECF anti-σ factor can also be released from the ECF σ factor by a phosphorylated response regulator NepR, this partner-switching mechanism has
recently be discovered in alphaproteobacteria [20]. Some ECF σ/anti-σ factors form together with an outer membrane TonB-dependent receptor, a trans-envelope signal transduction pathway [31]. These TonB-dependent receptors are involved in the uptake of specific molecules but also they sense and transmit, via the anti-σ factor, extracellular signals, which lead to the activation of a specific ECF σ factor.

In the present study, we investigated the role of the A. butzleri RM4018 ECF σ factors. We developed the genetic tools to manipulate A. butzleri and determined the regulons of most ECF σ factors using a combination of microarray-based transcriptome analysis and functional assays.

Materials and Methods

Bacterial Strains, Plasmids and Growth Conditions

Bacterial strains and plasmids used in this work are listed in Table 1. A. butzleri strains were routinely grown at 30 °C in Brain Heart Infusion (BHI) broth (Oxoid) or on Mueller Hinton (MH) agar (Oxoid) supplemented with 5% sheep blood (Biotrading). E. coli strains were routinely grown at 37 °C in Luria-Bertani (LB) broth or on LB agar plates (Biotrading) supplemented with ampicillin (100 μg/ml) or kanamycin (50 μg/ml) when needed.

Construction of A. Butzleri ECF σ Factor and ECF Anti-σ Factor Mutants

The genes encoding the seven ECF σ factors and their cognate anti-σ factors were amplified using the primer pairs AB1098F/AB9087R, AB1044F/AB1044R, AB1437F2/AB1437R2, AB1460F/AB1460R, AB1576F/1576R, AB2151F/AB2151R and AB2300F/2300R (Table 2), the proofreading enzyme Pfu (Promega) and A. butzleri RM4018 chromosomal DNA as template. The PCR products were tailed with a 5′-A nucleotide using Taq polymerase (Invitrogen) and ligated into the pGEM-T Easy vector (Promega) to obtain pGEMab0983-0984, pGEMab1040-1041, pGEMab1429-1430, pGEMab1452-1453, pGEMab1567–1568, pGEMab2164–2165 and pGEMab2315–2316 (Table 1). Inverse PCR was performed on the σ/anti-σ plasmids to delete a large part of both the σ and anti-σ factor (σ/anti-σ knock-out plasmids) or of only the anti-σ factor encoding genes (anti-σ knock-out plasmids). Unique BamHI restriction sites were introduced at the same time. The plasmid pGEMab1429–1430 was obtained by digesting plasmid pGEMab1429 with BamHI and BclI. The knockout constructs were created by digestion of the inverse PCR products with BamHI and ligation to a 1.4-kb BamHI fragment containing a kanamycin resistance gene (aph(3′)-I) from pMW2 [21].

The ECF σ factor genes were inactivated by marker exchange mutagenesis. First, the knock-out plasmids were introduced into A. butzleri RM4018 by electrotransformation. To obtain electrocompetent A. butzleri RM4018, a 5 ml overnight culture was diluted 20 times in 100 ml fresh BHI medium and incubated at 30 °C on a shaking platform (150 rpm). Bacteria were harvested by centrifugation (4,500 × g, 1 h, 4 °C) when the optical density at 550 nm had reached values between 0.2 and 0.6. The bacteria were washed twice in 5 ml of ice-cold sucrose-glycerol solution (15% glycerol; 272 mM sucrose in water), resuspended in 0.5 ml ice-cold sucrose-glycerol solution and aliquoted into 50 μl solutions containing approximately 3–5 × 10^5 CFU. One μg of each plasmid was added to 50 μl (approximately 3–5 × 10^6 CFU) of competent A. butzleri RM4018 and incubated for 3 min on ice. The cells were transferred to a 0.2 cm electroporation cuvette (Bio-Rad) and electroporated using a Bio-Rad Gene Pulser set at 2.25 kV, 400 Ω and 25 μF. Bacteria were recovered in 1 ml of BHI broth for 10 min at room temperature, transferred to 2 ml of pre-warmed BHI and incubated for 3 hours at 30 °C on a shaking platform (150 rpm). Mutants were selected by growing the cells for two to five days at 30 °C aerobically on kanamycin-containing MH plates. Homologous recombination resulting in double-crossover events was verified by PCR.

Phenotype Characterization

Growth curves were generated by diluting pre-cultures grown overnight in BHI to a starting optical density (OD_{550}) of 0.05 in 30 ml of BHI. The 30 ml cultures were grown in conical flask under aerobic conditions, 150 rpm at 30 °C. Bacterial growth and cell density were monitored by measuring the absorbance at 550 nm at different time intervals. The exponential growth rate was calculated from four separated growth experiments. Several stress conditions which limited the growth of the wildtype Arcobacter strain were tested. To measure the influence of extreme temperatures, the starting cultures were incubated at 4 °C or 60 °C for 15 min prior to their incubation at 30 °C. To study the effect of the pH on the growth of the strains, the pH of the BHI was adjusted to pH 5 or 9. Osmotic stress was tested by adding 0.35 M NaCl to the BHI. In additional experiments, other stress-inducing chemicals were added to the BHI to concentrations which limited the growth of the wildtype Arcobacter strain. These chemicals included: ethanol (5%); SDS (1%); the iron chelator 2,2-dipridyl (300 μM); the oxidative stress-inducing chemicals H_2O_2 (0.04%) and diamide (2 mM); or antimicrobial compounds such as penicillin G and polymyxin B (25 μg/ml). Motility assays were performed by stabbing the strains with a pipette tip into semisolid medium (thioglycolate medium containing 0.1% agar, Difco), followed by incubations under aerobic conditions at 30 °C or 37 °C for 48 hours.

RNA Isolation

Overnight grown cultures of A. butzleri were diluted to an OD_{550} of 0.1 in BHI and incubated at 30 °C on a shaking platform set at 150 rpm. RNA was isolated from 5 ml of mid-logarithmic phase cultures (OD_{550} of approximately 0.5), using the RNA-BeSM kit (Tel-Test, Inc) following the manufacturers specifications.

Microarray Hybridization and Analysis

For expression profiling, an indirect comparison of gene expression levels was performed [22,23]. In this microarray experimental design, each labeled cDNA was combined with labeled genomic DNA from A. butzleri RM4018. Mixtures were hybridized to a previously designed and manufactured A. butzleri DNA array [15]. Labeling of RNA and DNA, hybridization procedure and microarray data analysis were performed as previously described [24]. Details of the microarray have been deposited in the NCBI GEO repository (http://www.ncbi.nlm.nih.gov/geo/) under platform accession number GPL14948. The microarray data set has been deposited in the NCBI Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE34089.

Real-time RT-PCR

Primers in this assay were designed using the Primer Express software (Applied Biosystems) and are listed in Table 2. Prior to amplification, RNA samples were treated with RNase-free DNase I (Invitrogen). RT-PCR was performed on 0.2 μg of DNase I treated RNA with 1 μM of primers and the SYBR® Green I kit (Eurogentec) using a LightCycler® 480 Real-Time PCR System (Roche). The PCR parameters were 30 min at 48°C;
Table 1. Bacterial strains and plasmids used in this study.

| Bacterial strain or plasmid | Origin/function | Source |
|-----------------------------|----------------|--------|
| **A. butzleri strains**     |                |        |
| A. butzleri RM4018          | Human clinical isolate (ATCC 49616) | USDA* |
| A. butzleri Δσ7/Δn7::Km     | RM4018 derivative Δab0983–0984::aph(3')-III | This study |
| A. butzleri ΔAn7::Km        | RM4018 derivative Δab0984::aph(3')-III | This study |
| A. butzleri Δσ7/ΔAn7::Km    | RM4018 derivative Δab1040–1041::aph(3')-III | This study |
| A. butzleri ΔAn7::Km        | RM4018 derivative Δab1041::aph(3')-III | This study |
| A. butzleri Δσ7/ΔAn7::Km    | RM4018 derivative Δab1452–1453::aph(3')-III | This study |
| A. butzleri ΔAn7::Km        | RM4018 derivative Δab1453::aph(3')-III | This study |
| A. butzleri Δσ7/ΔAn7::Km    | RM4018 derivative Δab1567–1568::aph(3')-III | This study |
| A. butzleri ΔAn7::Km        | RM4018 derivative Δab1568::aph(3')-III | This study |
| **E. coli strains**         |                |        |
| E. coli DH5α NCCB2955       | Competent cells for cloning | NCCB** |

**Plasmids**

| plasmid                   | Origin/function | Source |
|---------------------------|----------------|--------|
| pGEM-T Easy               | Cloning vector, Amp' | Promega |
| pGEMab0983–0984           | pGEM-T Easy containing ab0983–0984 | This study |
| pGEMab1040–1041           | pGEM-T Easy containing ab1040–1041 | This study |
| pGEMab1429–1430           | pGEM-T Easy containing ab1429–1430 | This study |
| pGEMab1452–1453           | pGEM-T Easy containing ab1452–1453 | This study |
| pGEMab1567–1568           | pGEM-T Easy containing ab1567–1568 | This study |
| pGEMab2164–2165           | pGEM-T Easy containing ab2164–2165 | This study |
| pGEMab2315–2316           | pGEM-T Easy containing ab2315–2316 | This study |
| pGEMab0983–0984           | pGEM-T Easy containing ab0983–0984 | This study |
| pGEMab1040–1041           | pGEM-T Easy containing ab1040–1041 | This study |
| pGEMab1429                | pGEM-T Easy containing ab1429 | This study |
| pGEMab1453                | pGEM-T Easy containing ab1453 | This study |
| pGEMab1568                | pGEM-T Easy containing ab1568 | This study |
| pGEMab2164                | pGEM-T Easy containing ab2164 | This study |
| pGEMab2316                | pGEM-T Easy containing ab2316 | This study |
| pGEMab1040–1041::km       | pGEM-T Easy containing ab1040–1041::aph(3')-III | This study |
| pGEMab1429–1430::km       | pGEM-T Easy containing ab1429–1430::aph(3')-III | This study |
| pGEMab1452–1453::km       | pGEM-T Easy containing ab1452–1453::aph(3')-III | This study |
| pGEMab1567–1568::km       | pGEM-T Easy containing ab1567–1568::aph(3')-III | This study |
| pGEMab2164–2165::km       | pGEM-T Easy containing ab2164–2165::aph(3')-III | This study |
| pGEMab2315–2316::km       | pGEM-T Easy containing ab2315–2316::aph(3')-III | This study |
| pGEMab0984::km            | pGEM-T Easy containing ab0984::aph(3')-III | This study |
| pGEMab1041::km            | pGEM-T Easy containing ab1041::aph(3')-III | This study |
| pGEMab1429::km            | pGEM-T Easy containing ab1429::aph(3')-III | This study |
| pGEMab1453::km            | pGEM-T Easy containing ab1453::aph(3')-III | This study |
| pGEMab1568::km            | pGEM-T Easy containing ab1568::aph(3')-III | This study |
| pGEMab2164::km            | pGEM-T Easy containing ab2164::aph(3')-III | This study |
| pGEMab2316::km            | pGEM-T Easy containing ab2316::aph(3')-III | This study |
| pGEMab1041::km:Km         | pGEM-T Easy containing ab1041::aph(3')::Km | This study |
| pGEMab1429:Km             | pGEM-T Easy containing ab1429:Km | This study |
| pGEMab1453:Km             | pGEM-T Easy containing ab1453:Km | This study |
| pGEMab1568:Km             | pGEM-T Easy containing ab1568:Km | This study |
| pGEMab2164:Km             | pGEM-T Easy containing ab2164:Km | This study |
| pGEMab2316:Km             | pGEM-T Easy containing ab2316:Km | This study |

A. butzleri Extracytoplasmic Function σ Factors
Standard deviations were calculated and displayed as error bars. Each sample was examined in four replicates and was gene. Fold changes were calculated according to the equation in the reactions, the calculated threshold cycle (Ct) for each gene amplification was normalized to the Ct values for the g34A gene. Fold changes were calculated according to the ΔΔCt method [25]. Each sample was examined in four replicates and was repeated with at least two independent preparations of RNA. Standard deviations were calculated and displayed as error bars.

Nitrate/nitrite Assay
The nitrate reductase activity was determined by measuring the production of nitrite from nitrate as previously described [26]. Briefly, strains were grown overnight (16 h) under aerobic conditions in BHI broth containing 20 mM of sodium nitrate. Nitrite accumulation in the supernatant was detected by mixing 50 µl of the culture supernatants with 850 µl of 1% (w/v) sulphanilamide dissolved in 1 M of HCl and 100 µl of 0.02% (w/v) naphthylethylenediamine solution. After 15 minutes the formation of p-sulfobenzene-azo ∆-naphthylamine was measured at 540 nm. The amount of nitrite present in 50 µl culture supernatant was estimated using a nitrite standard curve. The nitrite production was adjusted to the total bacterial proteins present in the culture as estimated by using the BCA protein assay kit. To determine the nitrate concentration in Arcobacter culture supernatants the supernatants were 10 times diluted in BHI. The diluted culture supernatants were incubated with sulphanilamide and naphthylethylenediamine as described for the nitrite detection. Next a trace amount of zinc dust was added to the mixture. The zinc dust reduces nitrate to nitrite and a red color develops due to the formation of p-sulfobenzene-azo ∆-naphthylamine. After 2 minutes the zinc has settled on the bottom and 900 µl of the supernatant was transferred to a new tube and the absorbance of the red color was measured at 540 nm. The amount of nitrite present in 50 µl culture supernatant was estimated using a nitrite standard curve which was also treated with zinc dust. Reactions were measured four times and repeated with two independent cultures.

Sox Enzyme Activity Assay
Overnight cultures in BHI were washed once with Simons citrate agar and supplemented with 0.01% sodium pyruvate. Cells were incubated at 30°C under aerobic conditions for 48 hours. The strains which were able to use the citrate as carbon source increased the pH of medium, resulting in a color change of the medium from green to blue.

Statistical Analyses
Analysis of variance (ANOVA) was used to identify if any of the mutations significantly changed the growth rate of the Arcobacter strains. Statistical analyses were performed using the SPSS 19.0 statistical package program (SPSS Inc., Chicago, IL).

Results
Mutagenesis of the ECF σ Factor and Anti-σ Factor Encoding Genes
For each ECF σ/anti-σ pair, mutational inactivation of the anti-σ factor results in constitutive induction of the σ response, while mutational inactivation of the σ factor results in the down-regulation of ECF-dependent transcription [19,27]. This allowed us to study the function of the ECFs in A. butzleri strain RM4018 without knowing the specific signal(s) that activate them. In order to address the role of the seven ECF σ factors in A. butzleri RM4018, we inactivated either both the σ and the anti-σ factor, or the anti-σ factor alone. Since no suitable Arcobacter antibiotic resistance cassettes were available and the previously-used chloramphenicol cassette of Campylobacter coli could not be used since A. butzleri RM4018 is resistant to chloramphenicol, we replaced a large part of the ECF coding regions with a kanamycin resistance gene (aph(3’)-III) of C. coli [21]. After modifying the Arcobacter mutagenesis protocol described by Ho et al. [28], we were able to replace the ECF σ/anti-σ factor genes AB0986/0987 (σ5, Λσ5), AB1044/1045 (σ2, Λσ2), AB1460/1461 (σ1, Λσ1), AB1576/1577 (Λσ2, σ5) and AB2300/2301 (Λσ2, σ5) with the Km cassette. We also obtained single mutants in the anti-σ factor genes Λσ1, Λσ2, Λσ3, Λσ4 and Λσ5. All mutants contained the Km cassette in the same orientation as the ECF genes. As no suitable Arcobacter shuttle plasmids exist and no other antibiotic cassettes could be used, we were unable to perform complementation studies.

Phenotype Characterization
Comparison of the various mutants with the parent strain RM4018 revealed neither differences in bacterial shape or colony formation on sheep blood plates nor in growth rate in BHI minimal medium as statistically estimated by Anova F = 1,777 (P>0.05) (Figure 1A and B). A common role of ECF σ factors is to protect bacteria against external stress [16]. Exposure of the A. butzleri mutant strains to extreme temperatures (e.g., 4°C or 60°C), pH 5...
or 9, or sub-inhibitory concentrations of stress-inducing chemicals (e.g., ethanol, SDS, metal ion chelators EDTA or 2,2-dipyridyl, the oxidative stress-inducing chemicals H2O2 and diamide, or the antimicrobial compounds penicillin G and polymyxin B) did not yield significant growth or morphology differences (data not shown). Similarly, the motility of the mutants on semisolid medium was unchanged compared to the wild-type. These results indicated that the functions of the Arcobacter ECF σ factors might be different from those in other bacterial species.

Identification of ECFσ Factor-regulated Genes

To identify the genes regulated by the RM4108 ECF σ factors, RNA was isolated from wild-type and mutant strains grown to mid logarithmic phase and subjected to microarray-based transcriptome analysis. To obtain maximal ECF σ factor-dependent transcript differences, transcripts of each σ/Δσ mutant and its cognate Δσ mutant were compared. Genes showing more than a fourfold change in transcript levels were considered as ECF σ dependent. Based on this criterion, the ECF σ factors σ1, σ2, σ3, σ4, and σ5 were found to regulate 3, 65, 14, 42 and 72 genes, respectively (Tables S1 to S5). None of the ECF σ factors appeared to regulate its own transcription. Interestingly, more than thirty genes were regulated by two or more ECF σ factors (Table 3). In agreement with the phenotypic characterization, the identified ECF σ-dependent genes indicate that the ECF σ factors of A. butzleri regulate other genes than ECF σ factors in other bacterial species.

Genes Regulated by AB0986 (ECF σ1)

The transcriptome analysis revealed that σ1 activates the transcription of AB0986, encoding a putative TonB-dependent receptor protein, and down-regulates the transcription of AB1593 and AB0053, that encode a putative sodium: alanine symporter and a L-lactate permease, respectively (Table S1).

Genes Regulated by AB1044 (ECF σ2)

Based on the microarray data, ECF σ2 activates the transcription of 35 genes and caused a down-regulation of the expression of 30 genes (Table S2). All annotated gene products represent putative proteins. The sodium solute symporter AB0504 is the most strongly up-regulated gene product. Apart from this, ECF σ2 is predicted to control the transcription of the genetic loci: AB0343–AB0346, encoding a Nrf-type nitrite reductase; AB0353 to AB0359, coding for a Nap- type nitrate reductase and a C4-dicarboxylate transport system; AB0576–0577, encoding an aldehyde dehydrogenase and an uncharacterized protein; AB0494–AB0495, encoding an acetate kinase and a phosphate

Table 2. Oligonucleotides used in this study.

| Primer name   | DNA sequence (5′ → 3′) |
|--------------|----------------------|
| Mutant construction |                      |
| AB0986F       | AACAGTTGCGTATATAAAGCTAG |
| AB0986R       | TCTTTTTATACCAATGTTGCC |
| AB0986F-bamHI | AGGATCTGCTATTTAAGGTGAGTAAGTAAAGCTCTTA |
| AB0986R-bamHI | AGGATCCAGAGGTCATTTTCTCAAAAGG |
| Arcoanti1-bamHI | AGGATCCGCTAAAAGGTTAAACCTTC |
| AB1044F       | TAAGTATTTTGAAAACATCGTCG |
| AB1044R       | AACACATCTCTCCAAATTTGATATC |
| AB1044F-bamHI | TGATTCGAAAAGAGTTCAATAGGG |
| Arcoanti2-bamHI | AGGATCCCTATTTAAGGTGAGTAAGTAAAGCTCTTA |
| AB1437F2      | CTATGATTTTTTAAATTATATAGTA |
| AB1437R2      | ATGCTTAATATATTGAGTT |
| AB1437R-bamHI | AGGATCCCTTATTTAAGGTGAGTAAGTAAAGCTCTTA |
| Arcoanti3-bamHI | AGGATCTCAGATTTAAGGTGAGTAAGTAAAGCTCTTA |
| AB1460F       | ACTATATCAATATATTGAGTT |
| AB1460R       | AAAAATATCTATATTGAGTT |
| AB1460F-bamHI | AGGATCCGCTAAAAGGTTAAACCTTC |
| AB1460R-bamHI | CGGATCTCTTGACATATTGAGTT |
| Arcoanti4-bamHI | AGGATCCGCTAAAAGGTTAAACCTTC |
| AB1576F       | TGCTTAAATATTGAGTT |
| AB1576R       | TTTTCAACAGGACATTATCG |
| AB1576F-bamHI | TGATTCGAAAAGAGTTCAATAGGG |
| AB1576R-bamHI | TGATTCGAAAAGAGTTCAATAGGG |
| Arcoanti5-bamHI | AGGATCTCAGATTTAAGGTGAGTAAGTAAAGCTCTTA |
| AB1215F       | TTCACTATATTGAGTT |
| AB1215R       | TTTTCAACAGGACATTATCG |
| AB1215F-bamHI | TGATTCGAAAAGAGTTCAATAGGG |
| AB1215R-bamHI | TGATTCGAAAAGAGTTCAATAGGG |
| Arcoanti6-bamHI | TGATTCGAAAAGAGTTCAATAGGG |
| AB2300F       | ACAATACAGTTTTGGGCG |
| AB2300R       | TTCAATATATTGAGTT |
| AB2300F-bamHI | AGGATCCGCTAAAAGGTTAAACCTTC |
| AB2300R-bamHI | CGGATCTCTTGACATATTGAGTT |
| Arcoanti7-bamHI | AGGATCCGCTAAAAGGTTAAACCTTC |
| qRT-PCR       |                      |
| arconapTaqF   | TGACACATATTTAAGGGGAGAAAA |
| arconapTaqR   | GAAACATTGGTTGTTAAGGGGAGAAAA |
| arconapAtaqF  | GCCGCTGGCCTGTT |
| arconapAtaqR  | CGCCACCGCTCCTGG |
| arcosoxDtaqF  | TGGCAGAATTTGTGTTGAAG |
| arcosoxDtaqR  | GAACACAGAAGGAAAGCAAG |
| arcosoxAtaqF  | CAGTGCTAGATACAGTAAAGGGGAGAAAA |
| arcosoxAtaqR  | GCAACCTGCTCCTGTTCAACAA |
| Abu100ctAtaqF | GGTTAGTTGCGACCGGAGG |
| Abu100ctAtaqR | CGTCACACCTCTGGTCAACAA |
| Abu96tctCtaqF | ACAAGCAAGAAGCAAAAATCTT |
| Abu96tctCtaqR | AAATTGGAAGAATACACCTTGTAAGT |
| AB0988Ftaq    | ATAAAGAAGGATGTTGGG |

Table 2. Cont.

| Primer name   | DNA sequence (5′ → 3′) |
|--------------|----------------------|
| AB0988Rtaq   | CGGTTGAGTGGCTGTTGAAATTC |
| AB1462Ftaq   | TCTCAAGAATTTCAAAAGGATAAGT |
| AB1462Rtaq   | AAGCCTCAGCTTGTGAAATTTATAT |
| AB1573Ftaq   | ACAATCCTATACCTGTCATTTTT |
| AB1473Rtaq   | GTAGCTGGGAGAAAGCGACTTCAACTAC |
| arcograTaqF2  | AAATTGGAAGAATACACCTTGTAAGT |
| arcograTaqR2  | TTGATATCATACCTGTCATTTTT |
Genes Regulated by AB1460 (ECF $\sigma^4$)

According to the transcriptomics data, $\sigma^4$ activates 11 genes and caused a down-regulation of the expression of 3 genes (Table S3). The main up-regulated gene product is the putative TonB-dependent receptor protein (AB1462) encoded by the same locus encoding the $\sigma^4$/anti-$\sigma^4$ factors. Among the other genes up-regulated by ECF $\sigma^4$ arc: napHGA (AB0354 to AB0356), aceEF (AB1480–1481), ffdA (AB0297), mfsA (AB0345) and fdaA1 (AB1507) encoding, respectively, a part of a Nap-type nitrate reductase, two pyruvate dehydrogenase components, a fumarate reductase subunit, a cytochrome c552 nitrate reductase and a formate dehydrogenase subunit. A methyl-accepting chemotaxis protein (AB0602) is the most strongly down-regulated gene product.

Genes Regulated by AB1577 (ECF $\sigma^5$)

Identification of ECF $\sigma^5$-dependent genes by transcriptomics analysis revealed 16 up-regulated and 26 down-regulated genes (Table S4). The closely-linked TonB-dependent receptor gene AB1573, was the most strongly up-regulated. The putative TctABC transport proteins AB0102–AB0104, the carbonic anhydrase AB0107, the putative ammonia monoxygenase AB0108, and the ubiquinolcytochrome c oxidoreductase encoded by the petAB (AB2054–AB2055) genes are among the gene products up-regulated by $\sigma^5$. The genes fur2 and cspA, and the genetic locus AB1062–1066, which includes NADPH quinonereductase, NADPH:flavodoxinoxidoreductase, and NADPH nitroreductase, were found to be repressed.

Genes Regulated by AB2301 (ECF $\sigma^7$)

The transcriptome analysis revealed that $\sigma^7$ activates 37 genes and represses another 35 (Table S5). Almost all annotated genes repressed by $\sigma^7$ were activated by $\sigma^2$. The genes encoding the putative TctABC transport proteins (AB0103–0105) are the most up-regulated genes by $\sigma^7$, while the sox operon and AB0494–AB0495, encoding an acetate kinase and a phosphate acetyltrans-
ferase are the most down-regulated. Although it does not affect the transcription of its linked TonB-dependent receptor gene (AB2299), σ^7 activated two other TonB-dependent receptor genes AB0705 and AB1870.

The A. butzleri ECF σ Factors Regulate Parts of the Electron Transport Chain

Unlike the tonB genes which also appear to be regulated by ECF sigma factors in other bacterial species, we found that a large number of genes regulated by A. butzleri ECF σ factors are involved in the electron transport chain. To verify that the ECF σ factors indeed influence the electron transport chain, we first performed real-time RT-PCR on the ECF-dependent tonB, nap, sox and tet genes. We could confirm that the tonB genes AB0988, AB1462 and AB1573 are indeed up-regulated by ECF σ^7, respectively (Figure 2a, c and d). ECF σ^2 activates the nap and sox genes (Figure 2b) of which the latter are repressed by σ^7 (Figure 2c). The real-time RT-PCR results also confirmed that the tet genes are down-regulated by ECF σ^2 and up-regulated by σ^7 and σ^7 (Figure 2b, d and e).

To prove that the differences in nap, sox and tet transcripts result in phenotypic differences, we determined the nitrate reductase activity, the ability to oxidize sulfate, and the utilization of citrate in ECF mutants compared to the wild-type. As expected, none of the strains except A. butzleri Δσ^2::Km produced nitrite from the supplied nitrate in the presence of oxygen (Figure 3a). The constitutive expression of σ^7 in the case of Δσ^2::Km mutant led to nitrite production regardless of the presence of oxygen, indicating that ECF σ^2 up-regulates the Nap-type nitrate reductase. To investigate whether the altered transcription levels of the sox genes resulted in a change in Sox enzyme activity, all the strains were grown for 6 hours in AM1 medium supplemented with sulfite and the pH indicator phenol red. The mutants Δσ^2 and Δσ^7/Δσ^7 further induced the change in medium pH observed during growth of the parental strain, indicating that these mutants have an increased ability to oxidize reduced sulfur compounds.

Table 3. Genes regulated by two or more ECF σ factors.

| Functional class* | ORF* | Gene* | Transcription activated by | Transcription repressed by |
|-------------------|------|-------|---------------------------|----------------------------|
| Energymetabolism: anaerobic respiration | AB0345 | nrfA | σ^7 and σ^2 | |
| | AB0345 | napH | σ^7 and σ^2 | |
| | AB0355 | napG | σ^7 and σ^2 | |
| | AB0356 | napA | σ^7 and σ^2 | |
| | AB0297 | frdA | σ^7 and σ^2 | |
| Energymetabolism: aerobic respiration | AB1442 | hydA | σ^7 | |
| Energymetabolism: pyruvate dehydrogenase | AB1481 | aceF | σ^7 and σ^2 | |
| Energymetabolism: electrontransport | AB0357 | dctP | σ^7 | |
| | AB0357 | dctQ | σ^2 | |
| | AB0359 | dctM | σ^7 | |
| | AB0102 | tct | σ^7 | |
| | AB0103 | soxK | σ^7 | |
| | AB0104 | soxL | σ^7 | |
| Transport/binding proteins: carbohydrates, organic acids and alcohols | AB0504 | petA | σ^7 | |
| | AB0376 | ald | σ^7 | |
| | AB0107 | cynT1 | σ^7 | |
| Central intermediary metabolism: general | AB0563 | soxC | σ^7 | |
| | AB0566 | soxY | σ^7 | |
| | AB0567 | soxZ | σ^7 | |
| | AB0568 | soxA | σ^7 | |
| | AB0569 | soxB | σ^7 | |
| Small molecule metabolism: carbon compound degradation | AB0494 | ackA1 | σ^7 | |
| | AB0495 | pta | σ^7 | |
| | AB1553 | katG | σ^7 | |

*The functions of the encoded proteins and the AB numbers are indicated according to Miller et al. [15].

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These results confirmed that ECF σ² and σ⁷ regulate the Sox sulfur oxidation proteins in an opposite way. Finally, we investigated whether these strains are able to utilize citrate, which causes an increase in the pH and a color change of the medium when these bacteria are grown on Simmons agar. In agreement with microarray and real-time RT PCR results, all strains except the mutants ΔAσ² and ΔDs⁷ caused a color change of the media, indicating that the ΔAσ² and ΔDs⁷ mutants could not metabolize citrate (Figure 3c).

**Discussion**

Although *Arcobacter* has been classified as an emerging pathogen (ICMSF, 2002), knowledge on this organism is still limited. The genome sequence of *A. butzleri* strain RM4018 [15] revealed that, unlike other characterized members of the epsilon proteobacteria, this strain possesses seven putative extracytoplasmic function (ECF) σ factors. These σ factors are often involved in the regulation of virulence or stress-related genes in other bacteria. In the present study, we developed genetic tools to manipulate *A. butzleri* and used these tools to investigate the function of the putative RM4018 ECF σ factors. *A. butzleri* is resistant to many different antibiotics [15,29,30]; therefore, it was difficult to find a suitable antibiotic cassette to inactivate the ECF σ factor-encoding genes. Only a kanamycin resistance gene from *C. coli* [31] appeared to be functional in strain RM4018. By electroporation we were able to generate 10 different mutants and were able to inactivate 5 of the 7 ECF σ factors and to identify the corresponding regulons. Despite repeated attempts, we were unable to mutate the ECF σ factors AB1430 (σ³) and AB2165 (σ⁹), which may indicate that these factors are essential for bacterial growth under the conditions employed.

ECF σ factors are involved in a wide range of environmental responses in many bacterial species [18]. They are mainly divided into stress response ECF σ factors and iron-starvation ECF σ factors [32]. Our results indicate that the ECF σ factors of the strain RM4018 are neither involved in iron-starvation nor in the stress response. Furthermore, we found no evidence of positive regulation of their own transcription, as often found for other ECF σ factors [27]. Except for the tonB genes, the RM4018 ECF σ factors regulate completely different sets of genes. This seemingly atypical gene regulation in *Arcobacter* has also been noted for the regulation of the flagellar genes as *Arcobacter* lacks the σ factors Flia and RpoN, which in many bacterial species regulate flagellar biosynthesis [15]. So, in contrast to other bacteria, many conserved genes in *A. butzleri* appear to be regulated by a σ factor belonging to a different σ factor class; therefore, knowledge on transcription regulation of conserved genes described in other bacterial species cannot be simply extrapolated to *A. butzleri*.

In *A. butzleri* RM4018, 5 out the 7 ECF σ/anti σ-factor pairs are flanked by genes encoding putative TonB-receptor proteins. In other species, the ECF σ/anti σ factors form, together with an
outer membrane TonB-dependent receptor, a cell-surface signaling (CSS) system [33]. Transcriptome analyses showed that σ1, σ4 and σ5 regulate the expression of the TonB-dependent receptors encoded by the same loci, suggesting the putative presence of three potential trans-envelope signal transduction systems in *A. butzleri* RM4018. Most of the described CSS systems are involved in iron signaling and transport [34]. However, other functions have been described including for the *Ralstonia solanacearum* Prh-PhiIR system, which senses the presence of a plant cell-wall structure and initiates a regulatory cascade that induces the hypersensitive response and the transcription of pathogenicity genes [35]. The role of the three putative CSS systems in *A. butzleri* RM4018 remains to be elucidated, as the growth of mutants in these systems are not affected by iron limitation.

Transcriptome analysis, as well as functional assays, showed that *A. butzleri* ECF σ factors also regulate a number of genes involved
in the binding and transport of specific compounds, energy metabolism and sulfur oxidation. We showed that *Araerobacter* does not reduce nitrate under aerobic conditions (Figure 3A), although it does under anaerobic conditions (data not shown). The ECF σ² is involved in this environmental adaptation as the Δσ²::Km mutant produces nitrite under aerobic conditions. This may indicate that oxygen stress has an effect on ECF σ² activity.

Several genes must be indirectly regulated by the ECF σ factors as they were up-regulated in the ECF σ mutants. Interestingly and also seen in *Bacillus subtilis* [36], a number of these genes are regulated by more than one ECF σ factor, which indicates that they may be needed under different growth conditions. For example, σ5 and σ7 regulate the dctPQM and sox genes in an opposite way. The DctPQM proteins form a tripartite ATP-independent periplasmic transporter, which catalyzes the uptake of C₂₃-dicarboxylates like malate, fumarate and succinate in many aerobic bacteria [37]. The Sox proteins which are not found in other members of the *Campylobacteraceae* are involved in the oxidation of reduced sulfur compounds in sulfur, photo- and chemo-lithotrophic bacteria [38]. Similarly, we showed that σ⁵ and σ⁷ activate, while σ⁵ and σ⁷ caused a down-regulation of the expression of the tctABC genes encoding a tricarboxylic transport system. Despite this, only σ⁵ and σ⁷ appeared to have a significant effect on citrate utilization. In many bacterial species a two-component signal transduction system activated by citrate is responsible for the activation of the tctABC genes [39]. In *A. butzleri*, the putative TctABC system may also depend on the two-component system (AB0105–0106) located directly downstream of the putative tctABC genes. The expression of AB0105–0106 was not dramatically affected by σ⁵ and this may explain why citrate utilization was distinct between Δσ5/Ar5 and Δσ7/Ar7. All together, the genes regulated by the different *A. butzleri* ECF σ factors indicate that the ECF σ factors form a complex network of regulons that have a major role in regulating bacterial metabolism.

In conclusion, we have shown in this initial study that the ECF σ factors of *A. butzleri* control the transcription of a complex network of regulons that contain genes that are not commonly regulated by ECF σ factors family of proteins. These genes are mainly involved in the energy metabolism. In contrast to other eubacteria, many conserved genes in *A. butzleri* appear to be regulated by a σ factor belonging to a different σ factor class.

Supporting Information

Table S1 Genes identified by micro-array analyses which are more than fourfold up or down regulated by *A. butzleri* ECF sigma 1.

Table S2 Genes identified by micro-array analyses which are more than fourfold up or down regulated by *A. butzleri* ECF sigma 2.

Table S3 Genes identified by micro-array analyses which are more than fourfold up or down regulated by *A. butzleri* ECF sigma 4.

Table S4 Genes identified by micro-array analyses which are more than fourfold up or down regulated by *A. butzleri* ECF sigma 5.

Table S5 Genes identified by micro-array analyses which are more than fourfold up or down regulated by *A. butzleri* ECF sigma 7.

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Author Contributions

Conceived and designed the experiments: IMM CTP MMSMW. Performed the experiments: IMM RM SH LD IH. Analyzed the data: IMM CTP MMSMW. Contributed reagents/materials/analysis tools: CTP JvP WG WGM MMSMW.

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