APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2007, p. 5058–5065 Vol. 73, No. 15
0099-2240/07/$08.00+0 doi:10.1128/AEM.00453-07

Responses of Wild-Type and Resistant Strains of the Hyperthermophilic Bacterium *Thermotoga maritima* to Chloramphenicol Challenge▽†

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Received 27 February 2007/Accepted 3 June 2007

Transcriptomes and growth physiologies of the hyperthermophile *Thermotoga maritima* and an antibiotic-resistant spontaneous mutant were compared prior to and following exposure to chloramphenicol. While the wild-type response was similar to that of mesophilic bacteria, reduced susceptibility of the mutant was attributed to five mutations in 23S rRNA and phenotypic preconditioning to chloramphenicol.

Phylogenetic analysis of antibiotic resistance genes in bacteria suggests a process of exchange and continuous evolution in the microbial world (5, 6). Microbial antibiotic resistance studies have focused on mesophilic bacteria and not on microorganisms that inhabit biologically restrictive niches. Hydrothermal environments, arguably populated by the most primitive microorganisms, have been minimally impacted by human intervention and, as such, could provide useful insights into the development of antibiotic resistance mechanisms.

Chloramphenicol (CAM) is a natural competitive inhibitor of the peptidyl transfer reaction catalyzed by 23S rRNA (25). Genome-wide transcriptional responses to CAM and other translational inhibitors present surprisingly similar features, even among phylogenetically diverse groups of mesophilic bacteria (10, 36, 45, 57). This transcriptional response usually involves induction of genes related to translational machinery and purine biosynthesis and repression of amino acid biosynthesis and aminoacyl-tRNA synthases (10, 28, 45). CAM induces both bacteriostatic and bactericidal effects (58), including increased translation inaccuracy events in vivo (65), filamentation (61), cold shock response (19, 45, 68), transient oxidative response (2), polyamine production (2, 52, 53), and up-regulation of genes encoding ribosomal proteins (10, 19, 36, 45, 57). Resistance to this antibiotic is typically associated with modifications in 23S rRNA and cellular processes that reduce cytoplasmic CAM accumulation (11, 12, 29, 43, 47, 54).

Thermotoga maritima, an evolutionarily deeply branched marine hyperthermophilic bacterium, is intrinsically resistant to aminoglycosides (27, 37) but sensitive to CAM. Here, transcriptomes of wild-type (WT) *T. maritima* MSB8 and a CAM-resistant spontaneous mutant were examined to compare response mechanisms of the hyperthermophilic and mesophilic bacteria. *T. maritima* strains were grown anaerobically at 80°C on sea salts medium supplemented with cellobiose (10 mM) under an N_2 headspace (51). Growth was monitored by cell density determination using epifluorescence (acridine orange) microscopy (30, 31).

The resistant mutant (RM) was selected by successive passages with increasing concentrations of CAM (5 μg/ml to 500 μg/ml) and isolated by serial dilution of the culture to extinction (7). The MIC was determined by monitoring the optical density at 600 nm and by direct cell counts. Antibiotic thermostability was tested using an *Escherichia coli*-based bioassay; minimal thermal deterioration of CAM under the experimental conditions tested was noted (48). The RM strain was found to have a MIC (1 mg/ml) significantly higher than that for the WT (25 μg/ml). Sequencing (23S rRNA) of the RM strain was performed by the Integrated Biotech Laboratories, University of Georgia, Athens.

For CAM challenge in batch culture, a 16-liter Microgen fermentor (New Brunswick Scientific, Edison, NJ) was used; 8 liters of sea salts medium was prepared by heating to 100°C for 20 min. Prior to inoculation (2%) at 80°C, the medium was reduced with 10% (wt/vol) sodium sulfide. The culture was agitated at 200 rpm and sparged continuously with N_2 to maintain anaerobic conditions. WT and RM cultures were challenged during mid-exponential phase (2×10^7 cells/ml) with 100 μg/ml of CAM. Samples were taken immediately before the CAM addition and then 5 and 30 min after challenge.

Continuous cultivation of *T. maritima* was performed in a 2-liter flask at 80°C (51, 59). A 7-h seed batch culture preceded continuous operation (dilution rate of 0.42 h^{-1}). Mechanical and biological steady-state conditions were attained by allowing at least 5 reactor volume changes, at which time cell densities stabilized at approximately 10^8 cells/ml (51, 59). CAM was added anaerobically, such that the culture was immediately exposed to 100 μg/liter; simultaneously, CAM was added to the feed to 100 μg/liter. Samples (350 ml) for transcriptional profiling were obtained from both batch and continuous cultures, as described elsewhere (22). Development of the cDNA microarray for *T. maritima* and the associated experimental and statistical methodology have been described elsewhere (13, 31, 51).

Based upon the genomic sequence of the *T. maritima* 23S

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† Supplemental material for this article may be found at http://aem.asm.org/.

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▽ Published ahead of print on 8 June 2007.

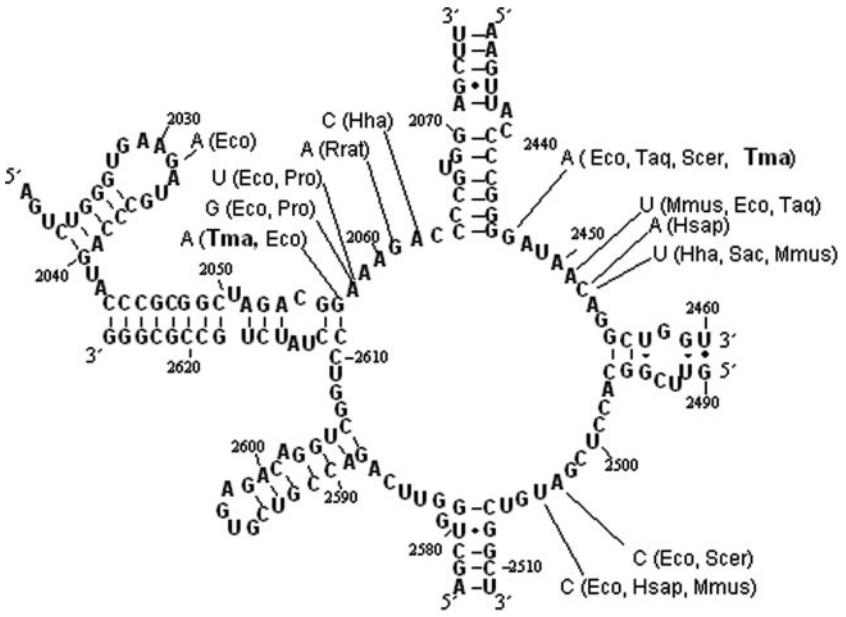

FIG. 1. Point mutations identified in the nucleotide sequence of 23S rRNA of a CAM-resistant mutant of *Thermotoga maritima* (Tma). Mutation sites are referenced to *E. coli* (Eco). Note that G2288A in *T. maritima* corresponds to an adenine at *E. coli* location 2169. Mutations identified in the 23S rRNA PTC loop related to CAM resistance are shown in Table 1 for *T. maritima* and other organisms/organelles: G2032A in *E. coli* (16); G2057A in *E. coli* (18) and *T. maritima* (this work); A2058G and A2058U in *Propionibacterium acnes* (Pro) (54) and *E. coli* (16); G2061A in *Rattus rattus* (Rrat) (35); A2062C in *Halobacterium halobium* (Hha) (40); G2447A in *E. coli* (64), *S. cerevisiae* (Scer) (17), *Thermus aquaticus* (Taq) (50), and *T. maritima* (this work); A2451U in *Mus musculus* (Mmus) (33), *E. coli* (64), and *T. aquaticus* (50); C2542A in *Homo sapiens* (Hsap) (9); C2542U in *H. halobium* (40), *Sulfolobus acidocaldarius* (Sac) (1), and *M. musculus* (60); A2053C in *S. cerevisiae* (17) and *E. coli* (70); and U2054C in *E. coli* (70) and *H. sapiens* (9, 33).

rRNA (44), the RM was found to contain five mutations (Fig. 1 and Table 1). Mutations to nucleotide G2176 (*E. coli* 23S rRNA nomenclature used for reference) have been associated with resistance to translational inhibitors in other mesophilic bacteria (18, 21, 56). The mutation corresponding to G2568A was initially discovered in CAM-resistant *Saccharomyces cerevisiae* (17). When engineered into *E. coli*, the corresponding mutation, G2447A, was associated with resistance to CAM (64), while for reconstituted ribosomes from *Thermus aquaticus*, this mutation triggered a peptidyl-transferase activity rate reduction ranging from 30% to 73% (50). The third mutation in domain V, G2288, located at the F (final) site of tRNA transit through the ribosome, corresponds to an adenine present at the same location in *E. coli* (position 2169). This constitutes a novel, perhaps compensatory, mutation in *T. maritima* (3, 38, 39), Given the localization at the F site (34), the G2288A mutation most likely affects the final release of the nonaminoacylated tRNA to the cytoplasm, possibly tuning ribosome function during translation to minimize the loss of peptidyltransferase activity arising from the double mutation (G2176A and G2568A) at the peptidyltransferase center (PTC) (49, 69).

TABLE 1. Mutations in the 23S rRNA PTC loop related to CAM resistance

Mutation location in *T. maritima*	Mutation location in *E. coli*	23S rRNA domain	Previously noted in other organism(s) (reference)
2980_2981 (*insG*)	2862_2863 (*insG*)	VI	No
G2568A	G2447A	V	Yes: *E. coli* (64), *T. aquaticus* (50), *S. cerevisiae* (17)
G2288A	A2169	V	No
G2176A	G2057A	V	Yes: *E. coli* (18)
G907U	G830U	II	No

CAM addition to WT batch cultures impeded growth for about 3 h, after which time growth resumed briefly, albeit at a reduced rate (Fig. 2A). In contrast, after a slight pause following CAM addition, the RM strain continued to grow at the same rate as before antibiotic challenge, which was about half that of the WT (64 min versus a 37-min doubling time [t_d]) (Fig. 2B). Morphological changes have been reported for mesophilic bacteria upon exposure to antibiotics (15, 23, 26, 55, 61). Here, within 30 min of CAM exposure, the WT was coccoidal, whereas the RM retained the characteristic *T. maritima* rod-shaped morphology. CAM challenge of the WT continuous culture led to washout but had no noticeable effect on the RM culture.

Differences between RM and WT transcriptomes prior to CAM challenge. Different transcriptomes have been observed for wild-type bacteria and corresponding vancomycin-resistant mutants, even in the absence of the selective agent (42). Similar characteristics were observed here for *T. maritima* prior to CAM exposure (RM00 versus WT00) (Table 2). In continuous culture, only 19 open reading frames (ORFs) were differentially regulated twofold or more in the RM strain relative to the WT, compared to 131 ORFs in batch culture. In batch culture, 32 ribosomal structural proteins (2/3 of the identifiable *T. maritima* ribosomal proteins) were represented within this group (44). Other translation-associated genes up-regulated in the RM strain prior to challenge in batch culture included

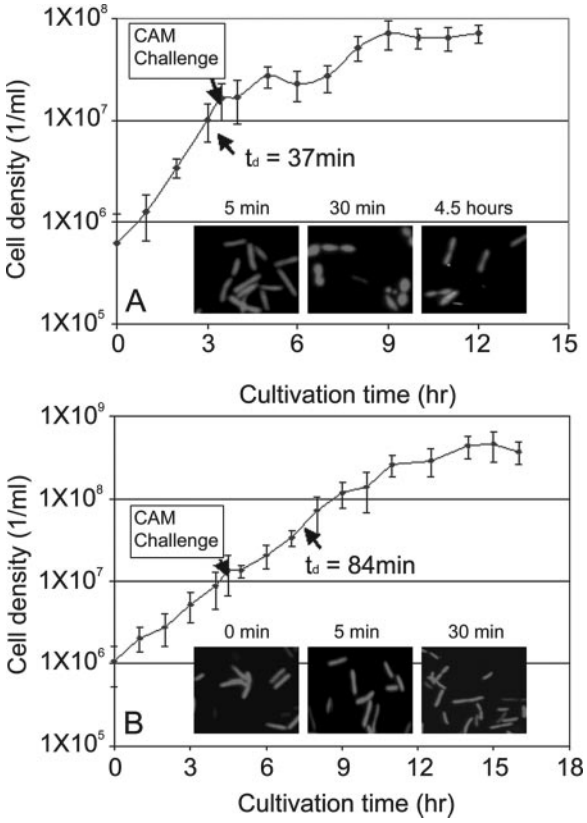

FIG. 2. CAM challenge of WT *T. maritima* (A) and the RM (B). The mutant strain grew more slowly than the WT prior to CAM challenge but showed no morphological changes after challenge compared to the response of the WT culture; WT morphology returned to the prechallenge state within several hours.

elongation factor (TM1502, TM1503, and TM1590), methionine aminopeptidase (TM1478), and SecY (TM1480) genes. Polyamine synthesis proteins (TM0654 to TM0656) and cold shock proteins CspC (TM1683) and CspL (TM1874) were also up-regulated prior to challenge, suggesting a preconditioning of the RM to offset the deleterious effect of CAM on RNA stability, protein translation, and oxidative stress.

Response of the WT to CAM challenge. Up-regulation of ribosomal proteins, transcription factors, and cold shock proteins has been implicated in the response of mesophilic bacteria to translational inhibitors (10, 36, 45, 57), and similar features were observed here for *T. maritima* (Tables 3 and 4). The WT response to CAM was immediate, with 274 and 256 ORFs differentially transcribed in batch and continuous cultures, respectively, within 5 min following CAM challenge (Table 2). Fewer ORFs responded during the 5- to 30-min period (36 for batch culture and 79 for continuous culture).

In bacteria, polyamines are associated with a variety of functions, including osmoregulation, response to pH, oxidative stress, growth rate, and induction of death in late stationary phase (4, 14, 32, 66, 73). In hyperthermophiles, polyamines stabilize nucleic acids at high temperatures (63) and are essential components of in vitro protein synthesis systems (37, 67). In *T. maritima* MSB8, a direct correlation between the presence of polyamines and response to high temperatures has been reported (74). Here, TM0654 (spermidine synthase) and TM0655 (*S*-adenosylmethionine decarboxylase) were up-regulated in the WT upon exposure to CAM (see Tables 3 and 4). Divergently transcribed from the spermidine synthase genes is a putative operon, comprised of TM0657 (rubrerythrin), TM0658 (neelaredoxin), and TM0659 (rubredoxin). TM0657 to TM0659 were up-regulated upon exposure to CAM, especially in continuous culture. TM0657 is related to the *Pyrococcus furiosus* rubrerythrin in a mix-branched phylogenetic tree of bacterial/archaeal rubrerythrins (71). The functional role of the *P. furiosus* homologs to TM0657 to TM0659 has been recently demonstrated in vivo in *E. coli* (24); in addition to NAD(P)H rubredoxin oxidoreductase, these genes are required to complete the detoxification pathway for reactive oxygen species in anaerobic microbes (24, 71). It is noteworthy that the antimicrobial effect of CAM is not always associated with direct binding to the PTC (8, 46). For example, despite structural differences between the archaeal and bacterial ribosomes (72), methanogens are sensitive to CAM due to the presence of the aryl nitro group in this antibiotic, a moiety that under anaerobic conditions acts as an oxidizing agent (8, 46). Thus, a direct oxidative effect of CAM on *T. maritima* ribosomes cannot be ruled out.

The connection between accumulation of mRNAs from ribosomal protein operons and challenge with translational inhibitors has been observed across all bacterial groups, including *E. coli*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, *Haemophilus influenzae*, and *Bacillus subtilis* (10, 19, 36, 45, 57). This response is usually accompanied by down-regulation of amino acid synthesis and up-regulation of transcription/translation factors (e.g., IF-3, GreA, EF-Tu, and NusA). In the case of the WT batch culture, this effect was quite clear. The response to CAM indicated accumulation of mRNAs for

TABLE 2. Summary of differentially transcribed ORFs for *Thermotoga maritima* chloramphenicol challenge

Culture	No. of ORFs with ≥2-fold change			Supplemental material source for detailed listing of ORFs
	Up-regulated	Down-regulated	Total	
Batch				
WT05 vs WT00	139	135	274	Table S4
WT30 vs WT05	7	29	36	Table S6
WT30 vs WT00	166	186	352	
RM05 vs RM00	32	28	60	Table S8
RM30 vs RM05	279	241	520	Table S10
RM30 vs RM00	216	184	400	
RM00 vs WT00	75	56	131	Table S2
RM05 vs WT05	218	212	430	Table S12
RM30 vs WT30	151	97	227	Table S14
Continuous				
WT05 vs WT00	125	131	256	Table S3
WT30 vs WT05	32	47	79	Table S5
WT30 vs WT00	81	102	183	
RM05 vs RM00	7	1	8	Table S7
RM30 vs RM05	0	5	5	Table S9
RM30 vs RM00	23	7	30	
RM00 vs WT00	7	12	19	Table S1
RM05 vs WT05	136	135	271	Table S11
RM30 vs WT30	74	80	154	Table S13

TABLE 3. Batch culture transcriptional response of selected ORFs before and after exposure to CAM in the *T. maritima* MSB8 WT and RM strains

Function and GeneID no.	Change (fold) of ORF before/after exposure to CAM					Annotation
	Before exposure (RM vs WT)	After exposure for:				
		WT		RM		
		5 min	30 min	5 min	30 min	
Ribosomal proteins						
TM0150	NC[b]	2.2	2.0	NC	NC	Ribosomal protein L32
TM0451	NC	2.3	2.1	NC	NC	Ribosomal protein L33
TM0452	NC	NC	2.2	NC	NC	Pre-protein translocase SecE
TM1451	NC	5.2	6.4	NC	3.7	RNA polymerase sigma A factor
TM1453–1458[a]	NC	3.3	2.3	NC	2.4	Ribosomal proteins
TM1480	2.6	NC	NC	NC	NC	Pre-protein translocase SecY
TM1471–1487[a]	2.1	1.6	NC	NC	2.4	Ribosomal proteins
TM1489–1505[a]	3.5	2.5	2.1	NC	1.9	Ribosomal proteins
TM1565	NC	2.0	2.7	2.8	NC	Signal recognition particle protein
TM1568	1.8	2.5	2.0	2.0	NC	16S rRNA processing protein
TM1569	NC	2.0	1.7	1.9	NC	tRNA guanine-N1 methyltransferase
TM1590–1593[a]	3.6	2.6	1.9	2.2	2.4	Ribosomal proteins/translation factor
TM1627	2.0	2.3	NC	1.8	2.1	General stress protein Ctc
Translation factors						
TM1502	3.5	1.8	2.5	NC	2.7	Translation elongation factor Tu
TM1503	2.6	1.6	2.0	1.9	NC	Translation elongation factor G
TM1590	3.8	2.9	1.8	1.7	3.0	Translation initiation factor IF-3
Polyamines/oxidative response						
TM0654–0659[a]	2.0	3.9	6.6	NC	1.6	Polyamine biosynthesis, rubrerythrin, neelaredoxin, rubredoxin
Defense mechanism						
TM0119	1.9	NC	NC	NC	2.1	Acetamidase, putative
TM0120	1.8	NC	2.2	NC	5.2	Oxidoreductase, putative
ATP synthase						
TM1609–1616[a]	NC	2.7 ↓[c]	3.2 ↓	NC	2.3 ↓	F_1 ATP synthase subunits
Purine biosynthesis						
TM1245–1251[a]	2.5 ↓	2.9 ↓	2.9 ↓	NC	NC	Purine biosynthetic pathway
Amino acid biosynthesis						
TM0545–0555[a]	NC	2.5 ↓	2.8 ↓	NC	↓ 2.1	Thr, Val, Leu, Ile biosynthesis
Sugar utilization						
TM1068	NC	2.1	1.9	NC	NC	α-Glucosidase
TM1195	NC	2.1	2.0	NC	NC	β-Galactosidase
TM1219–1223[a]	NC	2.2 ↓	2.0 ↓	1.8 ↓	2.3 ↓	β-Linked sugars utilization operon
TM1835	NC	2.0	2.9	NC	2.5	Cyclomaltodextrinase, putative
TM1840	NC	2.8	3.3	NC	2.9	α-Amylase
TM1845	NC	2.2	2.2	NC	NC	Pullulanase
DNA repair						
TM0266	NC	7.1	2.4	2.0	NC	DNA-binding protein, HU
TM0480	NC	2.0	2.4	NC	NC	Exonuclease ABC, subunit A
TM0604	NC	4.1	2.7	2.2	NC	DNA-binding protein
Cold shock proteins/chaperones						
TM0198	NC	2.1	2.5	NC	1.9	Clp protease, ATPase subunit
TM0505	3.3	3.9	NC	2.5	NC	GroES
TM0506	4.6	2.0	NC	2.1	1.6	GroEL
TM0571	NC	3.2	3.9	NC	NC	Heat shock serine protease
TM1683	2.8	4.9	4.1	NC	2.2	Cold shock protein
TM1874	3.1	3.4	5.8	NC	5.8	Cold shock protein
tmRNA						
TM0504	2.3	2.5	1.6	2.3	4.7	tmRNA/signaling peptide

[a] For operons, change (fold) is reported as the arithmetic average for all ORFs in the locus.
[b] NC, change was ≤1.5-fold.
[c] ↓, down-regulation.

TABLE 4. Continuous culture transcriptional response of selected ORFs before and after exposure to CAM in the *T. maritima* MSB8 WT and RM strains

Function and GeneID no.	Change (fold) of ORF before/after exposure to CAM					Annotation
	Before exposure (RM vs WT)	After exposure for:				
		WT		TM		
		5 min	30 min	5 min	30 min	
Ribosomal proteins						
TM0150	NC[b]	1.8	1.6	NC	NC	Ribosomal protein L32
TM0451	NC	1.7	NC	NC	NC	Ribosomal protein L33
TM0452	NC	1.6	1.7	NC	NC	Pre-protein translocase SecE
TM1451	NC	3.7	2.8	NC	NC	RNA polymerase sigma A factor
TM1453–1458[a]	NC	3.9	2.7	NC	NC	Ribosomal proteins
TM1480	NC	1.8	NC	NC	NC	Pre-protein translocase SecY
TM1471–1487[a]	NC	1.8	NC	NC	NC	Ribosomal proteins
TM1489–1505[a]	NC	NC	2.1	NC	NC	Ribosomal proteins
TM1565	NC	4.3	4.4	NC	NC	Signal recognition particle protein
TM1568	NC	3.2	2.3	NC	NC	16S rRNA processing protein
TM1569	NC	2.3	2.0	NC	NC	tRNA guanine-N1 methyltransferase
TM1590–1593[a]	NC	3.8	3.0	NC	NC	Ribosomal proteins/translation factor
TM1627	NC	3.5	3.2	NC	NC	General stress protein Ctc
Translation factors						
TM1502	1.7	NC	2.2	1.7	2.1	Translation elongation factor Tu
TM1503	NC	NC	2.4	NC	NC	Translation elongation factor G
TM1590	NC	4.1	2.6	NC	NC	Translation initiation factor IF-3
Polyamines/oxidative response						
TM0654–0659[a]	NC	3.7	5.1	NC	NC	Polyamine biosynthesis, rubrerythrin, neelaredoxin, rubredoxin
Defense mechanism						
TM0119	NC	NC	1.6	NC	1.6	Acetamidase, putative
TM0120	NC	NC	NC	NC	NC	Oxidoreductase, putative
ATP synthase						
TM1609–1616[a]	NC	2.1 ↓[c]	NC	NC	NC	F_1 ATP synthase subunits
Purine biosynthesis						
TM1245–1251[a]	NC	1.6 ↓	NC	NC	NC	Purine biosynthetic pathway
Amino acid biosynthesis						
TM0545–0555[a]	NC	2.3 ↓	NC	NC	NC	Thr, Val, Leu, Ile biosynthesis
Sugar utilization						
TM1068	NC	1.6	NC	NC	NC	α-Glucosidase
TM1195	NC	1.7 ↓	NC	NC	NC	β-Galactosidase
TM1219–1223[a]	NC	3.7 ↓	3.6 ↓	NC	NC	β-Linked sugar utilization operon
TM1835	NC	4.2 ↓	3.9 ↓	NC	NC	Cyclomaltodextrinase, putative
TM1840	NC	NC	2.2 ↓	1.6	1.7	α-Amylase
TM1845	NC	NC	NC	NC	NC	Pullulanase
DNA repair						
TM0266	NC	3.1	1.6	NC	NC	DNA-binding protein, HU
TM0480	NC	NC	NC	NC	NC	Exonuclease ABC, subunit A
TM0604	NC	5.8	3.4	NC	NC	DNA-binding protein
Cold shock proteins/chaperones						
TM0198	NC	2.6	1.8	NC	NC	Clp protease, ATPase subunit
TM0505	NC	3.4	1.6	1.8	2.8	GroES
TM0506	NC	4.5	NC	2.1	3.3	GroEL
TM0571	1.8	2.9	1.6	NC	NC	Heat shock serine protease
TM1683	NC	2.7	NC	1.6 ↓	3.0 ↓	Cold shock protein
TM1874	NC	3.1	2.8	NC	NC	Cold shock protein
tmRNA						
TM0504	NC	NC	NC	NC	NC	tmRNA/signaling peptide

[a] For operons, change (fold) is reported as the arithmetic average for all ORFs in the locus.
[b] NC, change was ≤1.5-fold.
[c] ↓, down-regulation.

ribosomal protein operons and down-regulation of genes associated with the biosynthesis of amino acids. Down-regulation of F_oF_1 ATP synthase structural components was observed, as was down-regulation of the cellobiose uptake transporter (TM1219 to TM1223); cellobiose was the primary carbon and energy source used here. The purine salvage and de novo synthesis pathways in the WT were down-regulated within 5 min after CAM challenge, a response previously noted in *B. subtilis* (20). In the WT, CAM-induced genes encoding transfer-messenger RNA (tmRNA), DNA repair, and cold and heat shock proteins were up-regulated.

Response of the RM to CAM challenge. Table 2 summarizes genome-wide differential transcription for the RM in batch and chemostat cultures at 5 and 30 min after CAM challenge. In continuous culture, the RM was relatively insensitive to CAM challenge at 5 min; only eight genes were differentially transcribed (seven up and one down). In fact, the overall RM response in continuous culture was limited, with only 30 ORFs responding (23 up and 7 down) over the 30-min period after CAM challenge. The insensitivity to CAM in the chemostat likely relates to the 10-fold-lower ratio of CAM to cells compared to batch culture.

At 5 min postchallenge in the RM batch culture, ribosomal and heat shock proteins responded. However, in contrast to the WT at 5 min, there was no response of cold shock genes (TM1683 and TM1874) and the overall transcriptional response was limited (60 ORFs: 32 up and 28 down). The impact of batch CAM challenge on the RM transcriptome was most pronounced between 5 and 30 min (520 ORFs: 279 up and 241 down). The basis for this delayed response is unknown but may reflect a difference in CAM affinity for its ribosomal targets and/or a mutation in cell wall permeability, as reported previously in *Burkholderia cepacia* and *H. influenzae* (11, 12, 54). Many of the differentially expressed transcripts in the RM are annotated as hypotheticals (see Table S10 in the supplemental material) and could not be assigned to any COG category or to a family of recognizable protein domains (41, 62).

Up-regulation of cold shock proteins was observed along with significant down-regulation of heat shock proteins, a reversal of the initial response. In the RM at 30 min, induction of the heat shock proteins DnaK, GroEL, and GroES was noted, similar to prechallenge. In mesophilic bacteria, chaperone overproduction can buffer negative fitness effects associated with deleterious mutations and this may be the case here (39).

Conclusions. These results add to the limited information available on the bacterial resistome. Although the data reported here are for a nonpathogenic species from an unusual habitat, certain features in common with antibiotic response in mesophilic bacteria were noted. Increasingly stringent selective pressure facilitated the selection of a resistant strain that exhibited directly adaptive mutations (five were noted in 23S rRNA) and probably yet undefined compensatory ones, resulting in a phenotype that was preconditioned to antibiotic challenge. It was interesting that ORFs encoding many hypothetical proteins were triggered in both the WT and RM upon antibiotic exposure, indicating that there is still much to be learned about this phenomenon.

This work was supported in part by grants from the NASA Exobiology, DOE Energy Biosciences, and NSF Biotechnology Programs. M.R.J. acknowledges support from a Department of Education GAANN Fellowship, and SBC acknowledges support from an NIEHS Bioinformatics Traineeship.

We acknowledge helpful discussions with James Brown at NCSU.

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