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A Spontaneous Point Mutation in the Single 23S rRNA Gene of the Thermophilic Archaeon *Sulfolobus acidocaldarius* Confers Multiple Drug Resistance

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Development of transformable vectors for thermophilic archaea requires the characterization of appropriate selectable marker genes. Many antibiotic inhibitors of protein biosynthesis are known to bind to rRNA; therefore, we screened 14 for their capacity to inhibit growth of the thermophilic archaeon *Sulfolobus acidocaldarius*. Carbomycin, celesticetin, chloramphenicol, puromycin, sparsomycin, tetracycline, and thiostrepton all inhibited growth by different degrees. Spontaneous drug-resistant mutants were isolated from plates containing celesticetin or chloramphenicol. Six mutants from each plate exhibited a C-2585-to-U transition in the peptidyl transferase loop of 23S rRNA (corresponding to C-2452 in *Escherichia coli* 23S rRNA). The single-site mutation also conferred resistance to carbomycin. The mutated 23S rRNA gene provides a potentially useful and dominant marker for a thermophilic archaeal vector.

The abundance of a special class of introns in the rRNA genes of thermophilic archaea, which sometimes encode "homing" endonucleases (9, 19) and are able to spread amongst archaeal extreme thermophiles (1), has encouraged us to develop a vector transformation system as a first step in performing genetic analysis of these organisms. The 23S rRNA gene was selected as a potential marker gene because many antibiotic inhibitors of protein biosynthesis have been shown, by studies of point mutations conferring drug resistance and RNA footprinting of drug-ribosome complexes, to interact with 23S rRNA (reviewed in references 8 and 16).

The thermophile *Sulfolobus acidocaldarius* was selected. It is fairly easy to culture and, like many extreme halophilic and hyperthermophilic archaea, exhibits only one set of large rRNA genes per genome (15), such that the phenotypic effects of spontaneous mutations are not masked by transcripts from other gene copies as in bacteria and eucarya. A similar strategy was used earlier for an extreme halophile, by which two point mutations, conferring antibiotic resistance (17, 22), were exploited to develop a plasmid vector system that could be used to introduce designed mutations into the single-copy rRNA operon of *Halobacterium halobium* (23). Like the extreme halophiles (22), the thermophilic archaea are relatively resistant to antibiotics (6). However, they are sensitive to some drugs, and spontaneous mutants showing resistance to celesticetin and chloramphenicol were isolated and point mutations in their 23S rRNA genes were identified.

Antibiotic sensitivity of hyperthermophiles. Archaea are generally less sensitive to ribosomal antibiotics than are bacteria and eucarya (6, 22); therefore, we tested a range of ribosomal drugs for their capacity to inhibit growth of *S. acidocaldarius* at five drug concentrations, in the range from 1 to 250 μg/ml. *S. acidocaldarius* was from Brock's isolate 98-3 from Yellowstone National Park (DSM 639) (3) and was grown aerobically at 70°C and pH 3 in mineral medium containing 0.1% yeast extract (Difco, Detroit, Mich.) (3). About 10^6 *S. acidocaldarius* cells were inoculated into 50 ml of media containing drugs at concentrations from 1 to 250 μg/ml. The generation time for the cells was about 5 h. A_{450} values of the growing cultures were measured after 5 days of growth at 70°C. The results were normalized to the absorbance values of cultures grown under the same conditions without antibiotics and are summarized in Table 1. Carbomycin, celesticetin, chloramphenicol, puromycin, sparsomycin, tetracycline, and thiostrepton strongly inhibited growth of wild-type cells, while anisomycin, clindamycin, erythromycin, kanamycin, lincomycin, and spectinomycin had little or no effect on growth, even at the highest drug concentration tested (250 μg/ml). To ensure that the antibiotics were active at the high growth temperature (70°C), they were tested at the same temperature, and under similar conditions, on the thermophilic bacterium *Thermus aquaticus* (4) (Table 1) grown in Castenholtz medium; only anisomycin, which does not normally inhibit bacterial growth (14), had no effect.

Isolation and sequencing of spontaneous mutants. *S. acidocaldarius* cells were grown to an A_{450} of 0.7 to 0.8, and a 0.5-ml culture (10^8 to 10^9 cells) was added to a melted top layer of Gelrite containing celesticetin (10 μg/ml) or chloramphenicol (200 μg/ml). The mixture was then stirred and poured onto prewarmed plates containing the antibiotics at the same concentrations (21). The plates were inverted and incubated in a sealed plastic bag at 70°C to minimize evaporation. Five to 20 colonies per plate appeared after 16 days, and a relatively low spontaneous-mutation frequency, for each drug, of 1 per 10^7 to 10^8 cells was found. Six colonies from Gelrite plates containing celesticetin (10 μg/ml) and six from Gelrite plates containing chloramphenicol (200 μg/ml) were selected and purified by suspension in liquid medium and replating. After being replated, the colonies were grown in liquid culture in the presence of celesticetin (10 μg/ml) or chloramphenicol (200 μg/ml) and 23S rRNA was isolated.

Most spontaneous rRNA mutations which confer resistance to antibiotic inhibitors of peptidyl transferase are located in the central loop of domain V of 23S-like rRNA (reviewed in reference 16). Therefore, we concentrated on sequencing this region of the *S. acidocaldarius* 23S rRNA by reverse transcription from appropriately placed primers. Dideoxy sequencing of

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TABLE 1. Influence of antibiotic inhibitors of protein biosynthesis on the growth of *S. acidocaldarius* and *T. aquaticus*

Antibiotic	Concn (μg/ml)	Degree of inhibition[a] with:		
		Wild-type *S. acidocaldarius*	Mutant *S. acidocaldarius*	Wild-type *T. aquaticus*
Anisomycin	250	None	None	None
Carbomycin	250	Strong	Weak	Strong
Celesticetin	20	Strong	Weak	Strong
Chloramphenicol	250	Strong	Weak	Strong
Clindamycin	250	None	None	Strong
Erythromycin	250	None	None	Strong
Kanamycin	250	None	None	Strong
Lincomycin	250	None	None	Strong
Puromycin	50	Strong	Strong	Strong
Sparsomycin[b]	1	Strong	Strong	Strong
Spectinomycin	150	None	None	Strong
Tetracycline	25	Strong	Strong	Strong
Thiostrepton	25	Strong	Strong	Strong

[a] None, growth at 90 to 100% of the control level; weak, growth at 80 to 90% of the control level; strong, growth below 25% of the control level. Cell cultures were grown for 5 days at 70°C under the conditions described in the text.

[b] Three derivatives were tested, benzyl-sparsomycin, pentyl-sparsomycin, and octyl-sparsomycin (28).

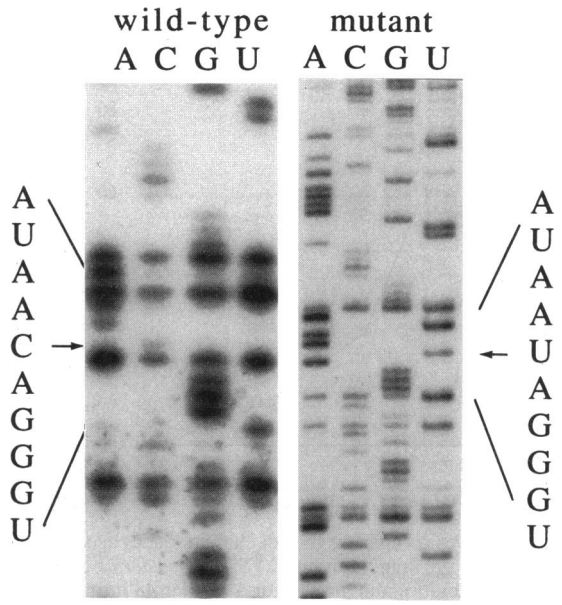

FIG. 1. Dideoxy sequencing of the 23S rRNA isolated from wild-type *S. acidocaldarius* (A) and a spontaneous chloramphenicol-resistant mutant of *S. acidocaldarius* (B). Arrows indicate the mutated position, 2585.

the 23S rRNA of *S. acidocaldarius* was performed, as described earlier (7), with the exception that the extension reaction was performed at 48°C and 8 μg of total RNA and 0.5 pmol of oligodeoxynucleotide primers labeled with ^{32}P at the 5′ end were used. The primer sequences were 5′-CCCTCCCACCTAC TCTACGC-3′, 5′-GCTGCACCTCCAGGGTGGG-3′, and 5′-CGACGGTCTAAACCCAGCTC-3′, which are complementary to positions 2248 to 2267, 2648 to 2666, and 2709 to 2728 of the 23S rRNA. Each of the 12 mutants was found to exhibit the single-nucleotide change C to U at position 2585 (C2585→U) (position 2452 by *Escherichia coli* numbering). The rRNA sequences of the wild type and a chloramphenicol-resistant mutant are compared in Fig. 1. No other sequence changes were detected in domain V of the 23S rRNA for any of the mutants.

Characterization of the mutants. Celesticetin and chloramphenicol sensitivities of wild-type cells and of three isolates of each type of spontaneous mutant were compared over a range of antibiotic concentrations, and growth characteristics as a function of antibiotic concentration are shown in Fig. 2. Growth of the wild type was completely inhibited at concentrations of celesticetin between 5 and 25 μg/ml, whereas the celesticetin-resistant strains exhibited high-level resistance at 25 μg/ml (Fig. 2A). Growth of the wild-type strain was reduced by one-half at a chloramphenicol concentration of 40 μg/ml and completely inhibited at 250 μg/ml, while the chloramphenicol-resistant isolates were strongly resistant at 250 μg/ml (Fig. 2B). Mutant strains isolated on celesticetin plates also yielded the same degree of resistance to chloramphenicol as did mutants isolated on chloramphenicol plates, and vice versa (data not shown).

The genetic stability of the mutants was tested by growing three drug-resistant clones, one isolated from celesticetin plates and the other two isolated from chloramphenicol plates, for 30 to 35 generations, in the absence of antibiotic, and no drug-sensitive revertants were detected.

The growth rates of wild-type and mutant strains were compared in the absence of drug in order to establish whether mutation of the universally conserved C-2585 produced any deleterious effects on cell growth. No changes in growth rate were detected, and no cellular defects were observed by light microscopy. This suggests that protein biosynthesis was not seriously impaired by the mutation.

The *S. acidocaldarius* mutant was tested with the other antibiotics, to investigate whether the mutation conferred resistance to them. The results are summarized in Table 1 and show that the C2585→U mutation only produced cross-resistance to carbomycin (Fig. 2C). Moreover, when a mutant isolated from a celesticetin plate was compared with one isolated from a chloramphenicol plate, the levels of carbomycin resistance were indistinguishable.

Conclusions. We have tested the ability of a collection of antibiotics, known to inhibit bacterial and/or eucaryotic ribosomes, to inhibit growth of the thermophilic archaeon *S. acidocaldarius*. A comparative study of the drug resistances of *S. acidocaldarius* and a thermophilic bacterium, *T. aquaticus*, grown at the same temperature (70°C), reinforced the view that archaea are resistant to many ribosomal inhibitors (6, 22), since anisomycin, clindamycin, erythromycin, kanamycin, lincomycin, and spectinomycin inhibited growth of the bacterium only. This could reflect either that the antibiotics cannot penetrate the cell membrane or that the ribosomal binding sites of the drugs have been altered; the lack of in vitro assays for *S. acidocaldarius* ribosomes renders it difficult to evaluate the latter possibility.

The C2585→U mutation in the single 23S rRNA gene of *S. acidocaldarius* confers resistance to celesticetin, chloramphenicol, and carbomycin (Fig. 3), and we believe that the point mutation is exclusively responsible for the drug resistance for the following reasons: (i) all 12 independent mutant isolates exhibited the same single transition in the central loop of domain V of the 23S rRNA; moreover, no other sequence changes were detected within domain V, which is considered to contain the primary RNA sites involved in peptidyl transferase and drug binding (reviewed in reference 16); (ii) mutants isolated on celesticetin plates were chloramphenicol resistant, and vice versa; (iii) mutants isolated from both celesticetin and chloramphenicol plates all exhibited the same level of resis-

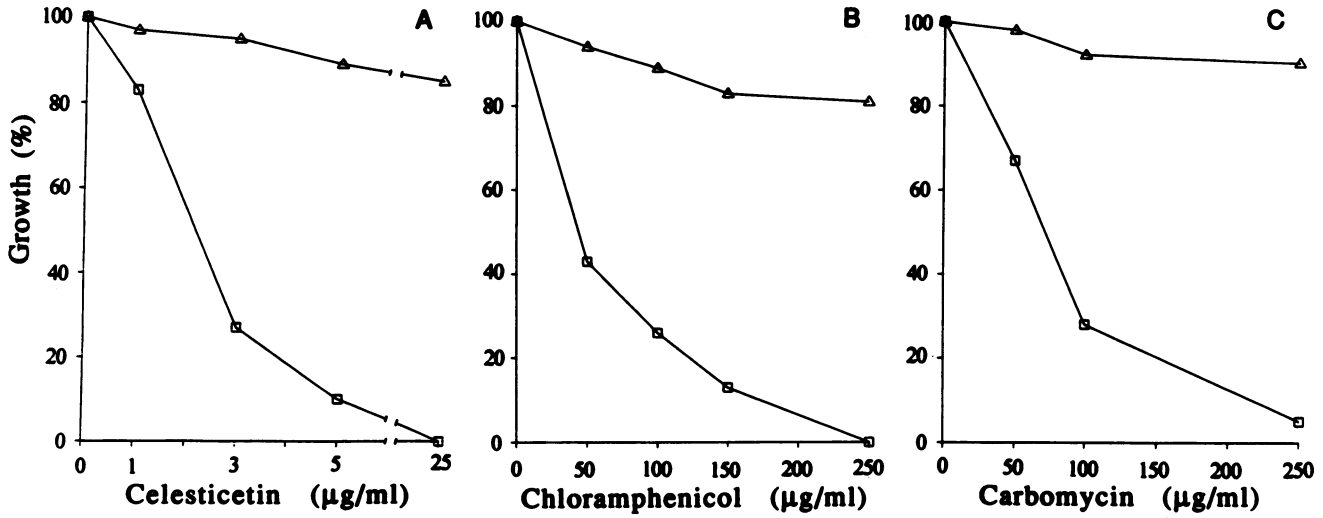

FIG. 2. Curves comparing growth characteristics as a function of the concentration of celesticetin (A), chloramphenicol (B), and carbomycin (C) for wild-type (□) and mutant (△) *S. acidocaldarius* cells. Cells were grown for five generations at 70°C and diluted to an A_{450} of 0.002 before addition of antibiotics and growth for a further 48 h under exponential growth conditions.

tance to carbomycin; (iv) the corresponding base change produced chloramphenicol resistance in *H. halobium* (22) and in mitochondria of higher eucaryotes (2, 26); and (v) each of the three antibiotics has been footprinted in the peptidyl transferase loop of *E. coli* 23S rRNA (11, 24) (Fig. 3).

The observed resistance of the U2585→C mutant to both celesticetin and chloramphenicol correlates with their competing for a common binding site on polysomes (25). The data suggest further that the lack of sensitivity of *S. acidocaldarius* to lincomycin and clindamycin (Table 1) reflects their inability to penetrate the cell membrane, because both drugs appear to have ribosomal (and polysomal) sites overlapping with those of celesticetin and chloramphenicol (10, 13). The mutation at C-2585 also serves to localize another potential interaction site of celesticetin on 23S rRNA in addition to A-2058 (*E. coli* numbering), which is N^6 monomethylated in *Streptomyces caelestis*, the producer of celesticetin (5).

The invariance of C-2585 among all sequenced large-subunit rRNAs suggests that it has an important functional role (12). It was surprising, therefore, that both the *S. acidocaldarius* mutant and a similar *H. halobium* mutant (22) grew normally in the absence of drug, while the corresponding *Tetrahymena thermophilia* mutant, which was anisomycin resistant, grew slowly, was cold sensitive, showed aberrant cell morphology, and was unable to mate (27). Possibly, defects in the archaea will be detected under more stressful growth conditions.

Finally, the mutant 23S rRNA gene characterized here should provide a useful marker for vector development for archaeal hyperthermophiles. It contains a single-site mutation within a conserved sequence that can easily be generated in other hyperthermophilic 23S rRNA genes; it occurs at a low frequency and is stable, with a very low level of revertants; and it has no apparent detrimental effect on cellular growth. It should also be dominant because the hyperthermophilic cells generally only contain one copy of the rRNA operon.

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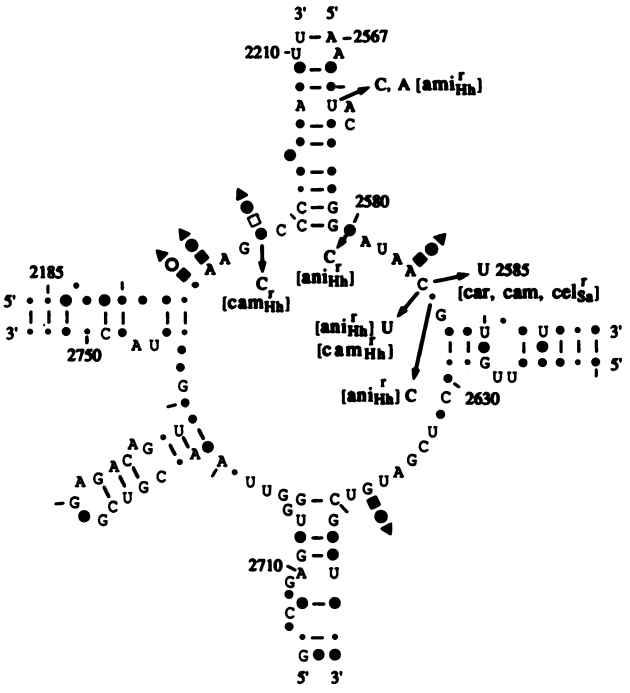

FIG. 3. Secondary structure of the central loop region of domain V of 23S rRNA, showing the mutated site, C-2585, in the spontaneous celesticetin- and chloramphenicol-resistant mutants. The structure is presented as a conservation plot, where letters denote nucleotides that are completely conserved amongst all the available sequences from archaea, bacteria, chloroplasts, and eucarya and filled circles of decreasing size represent nucleotides of decreasing levels of conservation from 99 to 25% (updated from reference 12). Open and filled symbols, adjacent to nucleotide positions, reflect rRNA footprinting data on complexes of *E. coli* ribosomes with celesticetin (□), chloramphenicol (○), and carbomycin (△) (11, 24). Filled symbols indicate drug-induced protection effects, and open symbols denote enhanced reactivities. Arrows show the locations of spontaneous single mutations which confer drug resistance on the archaeon *H. halobium* (Hh) (ami, amicetin; ani, anisomycin; cam, chloramphenicol; car, carbomycin; cel, celesticetin) (18, 20, 22).

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