Chromosomal marker exchange in the thermophilic archaeon *Sulfolobus acidocaldarius*: physiological and cellular aspects

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Exchange and recombination of chromosomal markers is an intrinsic genetic property of the thermoacidophilic archaeon *Sulfolobus acidocaldarius* that has not been thoroughly characterized. To clarify the mechanism and experimental usefulness of this process, the frequency of *S. acidocaldarius* prototrophs produced from mixtures of two pyrimidine auxotrophs under a variety of conditions was determined. The apparent efficiency of genetic exchange was essentially independent of the density of cells deposited on the surface of solid media. Furthermore, recombinant formation could initiate in liquid suspensions, as indicated by high recombinant frequencies resulting from mixtures plated at low cell densities, and the formation of recombinants at equal or higher frequencies in liquid suspensions that were never plated. Apparent initiation of genetic exchange in liquid at 22 °C was not prevented by DNase, prior digestion of parental cells with protease from *Streptomyces griseus*, or any other non-lethal chemical agent tested. The results support prior indications that chromosomal marker exchange in *S. acidocaldarius* proceeds via conjugation, and further indicate that this conjugation can initiate quickly in dilute liquid suspension. The mating system of *S. acidocaldarius* thus appears physiologically distinct from that of *Haloferax volcanii* but perhaps similar to conjugational transfer of *Sulfolobus* plasmid pNOB8. The frequency of recombinants formed in these assays (10^{-4} – 10^{-5} per c.f.u.) greatly exceeds the number of spontaneous forward mutational events per generation for biosynthetic genes in *S. acidocaldarius*. This suggests that chromosomal exchange has the potential to influence the genetic dynamics of natural *Sulfolobus* populations.

Keywords: geothermal environments, archaea, genetic exchange, cellular mechanism, conjugation

INTRODUCTION

Molecular phylogenetic analyses have shown that a group of diverse prokaryotes represents an evolutionary lineage distinct from that of bacteria. Members of this lineage, termed archaea (formerly 'archaeobacteria'; Woese *et al.*, 1990), apparently share a non-bacterial common ancestor with eukaryotes (Brown & Doolittle, 1995). As would be predicted from their great evol-

utionary age, the archaea exhibit unusual biochemical features and a remarkable ecological diversity (Jones *et al.*, 1987). They are particularly noted for dominating habitats defined by extremely harsh conditions. The most halophilic (Jones *et al.*, 1987), the most acidophilic (Schleper *et al.*, 1995b) and the most thermophilic (Stetter *et al.*, 1990) organisms known are, in each case, archaea. By the criterion of rRNA sequences, new yet uncultivated taxa of archaea appear to be abundant in milder, more extensive habitats such as subsurface ocean water (Hershberger *et al.*, 1996). These results suggest that archaea make up a significant proportion of the

Abbreviation: MPN, most probable number.

earth's microbiota and represent some of the most primordial organisms yet identified by sequence analysis (Barns *et al.*, 1994).

The extreme conditions which many archaea require for optimal growth, combined with their phylogenetic position relative to bacteria and eukaryotes, raise many questions regarding cellular function at the molecular level. *Sulfolobus* spp., for example, grow optimally at about 80 °C and pH 3; this combination of extremely high temperature and low pH efficiently denatures the macromolecules of mesophilic organisms. Thermostabilities of proteins and nucleic acids from *Sulfolobus* spp. and other thermophilic archaea have been studied *in vitro* by various research groups. However, properties of the organisms themselves raise many questions regarding cellular function at extremely high temperature which must be answered using direct, *in vivo* techniques. Development of genetic methods is especially strategic, and can be expected to depend heavily on identifying, analysing and exploiting natural genetic processes of these unusual prokaryotes.

Among thermophilic archaea, the greatest number of genetic processes occur in members of the genus *Sulfolobus*; for example, spontaneous mutation (Jacobs & Grogan, 1997), UV-induced mutation (Wood *et al.*, 1997) and photoreactivation (Grogan, 1997) have been studied in *Sulfolobus acidocaldarius*. Moreover, *S. acidocaldarius* spontaneously exchanges and recombines chromosomal genetic markers (Grogan, 1996a). This latter phenomenon seems to resemble the mating process of *Haloferax volcanii* (Mevarech & Werczberger, 1985), an archaeon otherwise unrelated to *S. acidocaldarius* which grows optimally at about 40 °C in concentrated NaCl solutions (Mullakhanbhai & Larsen, 1975). In both cases, mixtures of stable auxotrophs form prototrophs only if live and genetically distinct cells are allowed to come into contact, but plasmid dependence, unidirectional transfer and entry exclusion have not been observed (Mevarech & Werczberger, 1985; Rosenshine *et al.*, 1989; Rosenshine & Mevarech, 1991; Grogan, 1996a). This suggests that both of these archaeal exchange phenomena differ mechanistically from most examples of bacterial conjugation, which exhibit the latter three features (Clark & Warren, 1979; Willets & Skurray, 1980; Hopwood & Kieser, 1993; Macrina & Archer, 1993). It should be noted, however, that (i) a few examples of bacterial conjugation lack at least one of these 'classical' features, and (ii) unidirectional transfer of at least one genetic element, namely *Sulfolobus* plasmid pNOB8, has been observed in archaea (Schleper *et al.*, 1995a).

Of the three known examples of conjugation among archaea, those of pNOB8 and *H. volcanii* have been characterized to limited extents cytologically, and the results indicate major differences with respect to the cell-cell interaction necessary for transfer. Plasmid pNOB8 can spread rapidly through liquid cultures of recipient cells even under continuous agitation (Schleper *et al.*, 1995a), whereas agitation in liquid medium

prevents and disrupts genetic exchange in *H. volcanii* (Rosenhine & Mevarech, 1991). Indeed, *H. volcanii* mating requires incubation of a heavy film of cells on a solid support (Mevarech & Werczberger, 1985) and seems complete only when these physically stable contacts are maintained for several hours (Rosenhine & Mevarech, 1991).

Both of these situations have precedents among bacteria. Efficient DNA transfer between bacteria in liquid media, exemplified by the *Escherichia coli* fertility (F) plasmid, depends upon specific molecular recognition between donor and recipient cells that is mediated by surface proteins, i.e. the pili of Gram-negative bacteria (Clark & Warren, 1979; Willets & Skurray, 1980) or the adhesins of Gram-positive cocci (Clewell, 1993). The resulting high affinity of donor and recipient cells for each other causes rapid pairing of the two cell types upon mixing, effectively synchronizing genetic exchange and facilitating experimental analysis of its progress. Transfer of pNOB8 seems consistent with this type of conjugation, in the sense that mixed aggregates are reported to form rapidly when donor and recipient cells are combined (Schleper *et al.*, 1995a). In contrast, certain bacterial plasmids transfer only when donor and recipient cells are incubated in contact as a film on a solid support, in some cases for periods of time corresponding to several cell divisions (Hopwood & Kieser, 1993; Macrina & Archer, 1993). This requirement, which among Gram-negative bacteria correlates with the absence of a particular type of pilus (Wilkins, 1995), apparently reflects failure to establish cell pairs with high affinity, despite the full functioning of subsequent DNA transfer processes. Transfer of chromosomal markers between *H. volcanii* strains seems to resemble this latter bacterial situation.

The available data (Grogan, 1996a) do not indicate whether exchange of chromosomal markers in *S. acidocaldarius* more resembles the pNOB8 system or the *H. volcanii* system with respect to the physical and cellular parameters required for effective transfer. As a means to better understand its mechanism and to further define its practical aspects, we assayed marker exchange in *S. acidocaldarius* under a variety of conditions. The results suggest that, although chromosomal markers are exchanged and recombined as in *H. volcanii*, the cellular properties of *S. acidocaldarius* mating more closely resemble those of *Sulfolobus* plasmid pNOB8.

METHODS

Strains and growth conditions. The pyrimidine auxotrophs DG29 (*pyrD1*) and DG38 (*cbp-2*) were used in most experiments. Typical revertant frequencies for these two strains are about 3×10^{-7} revertants per c.f.u. (Wood *et al.*, 1997). In some experiments, the independent *pyrF* mutants MR27, MR39 and MR41 were used as parental strains to eliminate the potential of cross-feeding. Typical revertant frequencies for these strains are less than about 1×10^{-7} revertants per c.f.u.

All cultures to be mated were grown from a single colony in

xylose/tryptone/uracil ('XTura') medium (Jacobs & Grogan, 1997) with continuous agitation at 80 °C, unless otherwise noted. Cultures were harvested at cell densities less than about 4×10^8 cells ml⁻¹. Growth medium was removed by pelleting the cells, carefully decanting the supernatant, and resuspending the cell pellet in *Sulfolobus* dilution (Sdil) buffer (Grogan, 1996a) to achieve the desired cell density (1×10^9 cells ml⁻¹, unless otherwise noted).

Mating assays on solid medium. Cells (5×10^7 of each strain, unless otherwise noted) were mixed and spread on 6-cm-diameter plates of Sdil buffer solidified with gellan gum and incubated for 20 h at 75 °C. The cells were then quantitatively recovered from the plate surface by resuspension in three successive 1 ml aliquots of Sdil, using a sterile glass spreader, and plated to enumerate viable and recombinant cells (see below). Corrections for mating events subsequent to incubation on these plates were provided by control plates in which the two parental strains were deposited on separate sectors of one such plate but were otherwise processed in the same manner, as described previously (Grogan, 1996a).

The effects of cell density under these conditions were determined in two ways. The first was by varying the number of cells mated (from 1×10^7 to 4×10^8 of each parental strain) on an area of 10 cm². The other was by varying the surface area (from 0.3 to 20 cm²) upon which 5×10^7 cells of each parental strain were mated.

The effect of incubation time on solid medium was determined by preparing a series of identical mating and control plates and incubating them all at 75 °C. One mated and one control plate were then withdrawn at various times from 1 to 30 h, and the viable and recombinant c.f.u. of each were enumerated.

The expected number of random cell-cell contacts on solid media was estimated using the Poisson distribution of nearest neighbours in two dimensions, $P(d) = 1 - e^{-\pi\rho d^2}$, where $P(d)$ is the probability that two or more randomly dispersed points will lie within a distance d of each other, and ρ is the mean density of particles on the surface. For our purpose, d was set equal to 1 µm, which approximates the diameter of an *S. acidocaldarius* cell, and thus the centre-to-centre distance of two cells in contact.

Mating assays in liquid medium. The effects of cell density and of parental cell ratios were determined by incubating mixed suspensions of cells in Sdil buffer in 13-mm-diameter glass tubes containing 0.5 ml solidified Sdil. Correction for subsequent mating events was made by controls in which pure parental suspensions were similarly incubated and then combined at 22 °C shortly before enumeration. Cell density was varied either by varying the volume of Sdil buffer in which 8×10^6 cells were suspended [from nearly zero (film dried on surface of solid medium) to 1.5 ml] or varying the number of cells resuspended in 1 ml Sdil (from 4.8×10^8 to 6.4×10^8). In all other liquid matings, cells suspended in Sdil were simply combined in 13-mm-diameter glass culture tubes and treated as described in Results.

Enumeration. Unless otherwise noted, recombinant frequencies are Pyr⁺ colonies per 10⁶ c.f.u. plated. Aliquots (50–100 µl) of undiluted cell suspensions were plated on xylose/glutamine/casein hydrolysate medium and incubated for 1 week at 75 °C to select Pyr⁺ recombinants. The suspensions were also diluted in Sdil and plated on solid XTura medium to enumerate c.f.u. In one series of experiments, Pyr⁺ recombinants and total c.f.u. were estimated by the most probable number (MPN) method using liquid media.

(For this purpose, Dextrin-10 (Serva) was substituted for D-xylose as the primary carbon source.) Cell mixtures (0.2 ml each) were diluted serially in U-bottom polystyrene microdilution plates; growth was scored after incubation for 1 week at 75 °C.

RESULTS

Effect of cell density on efficiency of plate matings

To manipulate and analyse genetic exchange in *S. acidocaldarius*, we were interested in identifying conditions that interfere with the process. The initial hypothesis of this study was that, like *H. volcanii*, *S. acidocaldarius* conjugation should require cell-cell contacts to be established and stabilized on the surface of solid media. One prediction of this hypothesis is that conjugation should be effectively prevented even on solid media (which are used to enumerate recombinants) if mixtures were plated at sufficiently low cell densities. Results of prior plate-mating assays and their controls (Grogan, 1996a) seemed consistent with this hypothesis.

To measure the hypothesized density dependence, *S. acidocaldarius* pyrimidine (uracil) auxotrophs DG29 and DG38 were mixed and incubated on solid, non-nutritive medium as described previously (Grogan, 1996a), except that the mean cell density was varied nearly 600-fold, i.e. from 6×10^5 to 3.5×10^8 cells cm⁻². The efficiency of genetic exchange, measured as the ratio of Pyr⁺ recombinants to c.f.u., showed relatively little dependence on cell density, either overall or within a given trial (Fig. 1). (For reference, mean cell densities

Fig. 1. Efficiency of genetic exchange as a function of plating density. Strains DG29 and DG38 were mated on solid, non-nutritive medium; the surface density of parental cells was varied as described in Methods. Each symbol represents the mean of two determinations corrected for reversion plus mating events subsequent to the above incubation. Each symbol type (connected by lines) represents an independent trial. Recombinant frequencies are expressed as Pyr⁺ colonies per 10⁶ c.f.u. (note the compressed, logarithmic scale for cell density).

Table 1. Effect of cell compaction on the efficiency of genetic exchange

Matings were performed for 20 h at 75 °C in glass tubes containing solid medium (see Methods), using 1×10^8 cells of each parental strain. In most tubes (see first footnote below), the cells were suspended in a total volume of 1 ml.

Mating conditions*	Corrected recombinant frequencies†	'Background' subtracted‡
Film on surface	63, 153	40
Submerged cell pellet	89, 75	32
Non-agitated suspension	123, 33	33
Infrequently agitated	37, 50	30
Continuously agitated	66, 117	18

* 'Non-agitated' treatment corresponds to standard conditions used in other experiments. Modified conditions were: 'film on surface', cells were concentrated, deposited as a film on the surface of the solid medium, and left dry (as on a plate); 'submerged cell pellet', cells were pelleted against the solid medium from a mixed suspension (1 ml) and not resuspended; 'infrequently agitated', cell suspensions were vigorously agitated by hand at three different times separated by intervals of several hours; 'continuously agitated', cell suspensions were rotated at about 10 r.p.m., as described in Methods.

† Values are Pyr⁺ colonies per 10^6 c.f.u. (duplicate determinations) after correction for controls (see footnote below).

‡ Controls for recombination not attributable to the experimental conditions. The parental strains were incubated separately under the above experimental conditions, and then were combined at 22 °C shortly before plating to enumerate recombinants. The values in this column have been subtracted from the recombinant frequencies of duplicate determinations to yield the corrected recombinant frequencies shown in the preceding column.

below about $7 \times 10^6 \text{ cm}^{-2}$ should give less than 10% cells in contact on the plate surface if randomly distributed.) The results of Fig. 1 therefore do not support the hypothesis that *S. acidocaldarius* mating depends on the random formation of cell-cell contacts by adsorption of dense films of cells to an interface.

In an alternative test of the role of cell density, we compared the effect of incubating cells under various states of compaction. Matings were performed in tubes containing solid medium and 1×10^8 cells of each parental strain. At one extreme, cells were deposited as a film on the dry surface (about 0.8 cm^2) of the medium; at the other extreme, cells were suspended in 1 ml buffer in contact with the solid medium and kept continuously in suspension by rotating the tubes about an axis inclined 15° from horizontal. Intermediate treatments were: cell pellets incubated under 1 ml buffer, non-agitated 1 ml suspensions, and 1 ml suspensions vigorously shaken intermittently during incubation. All these treatments yielded comparable efficiencies of genetic exchange (Table 1), indicating that genetic exchange in *S. acidocaldarius* is not assisted by immobilization or dense packing of cells.

Fig. 2. Efficiency of genetic exchange in liquid medium. Cells were mated as non-agitated suspensions in Sdil buffer; cell density was varied as described in Methods. Other conditions were as for Fig. 1.

When cell mixtures were mated at 75 °C in liquid suspension, the efficiency of recombinant formation was not a decreasing function of cell density; in fact, increases were seen at the low cell densities (Fig. 2). Moreover, we noted that in some experiments (e.g. Table 1) 'non-mated' controls, which were subtracted as corrections in calculating the effects of the various test conditions, had yielded Pyr⁺ frequencies approaching those of the mated mixtures and about 100 times the typical revertant frequency of these strains (Table 1). Since these controls represent pure parental suspensions combined only shortly before enumeration of recombinants, these results raised the possibility that cell-cell contacts not only form in liquid suspension, but form rapidly at 22 °C.

In attempts to estimate the kinetics of this apparent room-temperature initiation of conjugation, we combined aliquots of parental cell suspensions in glass culture vessels, incubated the mixtures for various times at either 22 or 75 °C, and then enumerated Pyr⁺ recombinants and c.f.u. by plating. The results (Table 2) suggested that the apparent initiation of conjugation at 22 °C occurred very quickly, i.e. during the few minutes in which a mixed suspension was sampled and plated on solid medium. Significant increases above this basal level of conjugation were seen only after overnight incubation at 75 °C (Table 2), suggesting the need for biosynthesis or other metabolism to yield yet more recombinants. Supplementation of similar mating mixtures with uracil did not decrease the Pyr⁺ frequency, suggesting that revertants are not selected during prolonged incubation at 75 °C.

The rapidity of the presumed cell pairing at room temperature was unexpected, and prompted us to consider alternative explanations. In principle, we could not categorically eliminate initiation of conjugation on

Table 2. Genetic exchange efficiency as a function of time in mixed liquid suspensions

A total of 10^7 cells each of strains DG29 and DG38 were combined in a volume of 1.0 ml in 13-mm-diameter glass tubes (22 °C incubation) or screw-cap vials (75 °C incubation). After the indicated times, aliquots were withdrawn for enumeration of c.f.u. and recombinants.

Time of incubation (h)	Pyr ⁺ per 10^6 c.f.u. (% initial value)			
	22 °C		75 °C	
	Trial 1	Trial 2	Trial 1	Trial 2
0*	158 (100)	31 (100)	121 (100)	50 (100)
0.5	29† (18)	17 (53)	53† (44)	31 (62)
1.0	26 (17)	34† (108)	107 (88)	62† (125)
22	44 (28)	20 (65)	318 (262)	143 (288)

* For 0 h treatments, the actual time elapsed between initial mixing and full absorption of the liquid into the plates was about 2 min.

† In these cases, c.f.u. were taken to be the mean of the previous and subsequent time points.

solid media, since our mating assays for *S. acidocaldarius*, as well as those for *H. volcanii* (and for microorganisms generally), involve the prolonged incubation of cell mixtures on selective plates. However, the hypothesis that many cell contacts first form on the selective plates seemed unlikely in view of the low densities, i.e. a few million cells spread over about 30 cm² of selective medium. As predicted by random distribution, only 0.003% of the cells would be in contact with another cell under these conditions. Furthermore, density dependence of cell contacts on plates at cell densities comparable to those used in the present study has been used to prevent initiation of conjugation of *Enterococcus faecalis* (Ike & Clewell, 1984). In conjugational transfer of *E. faecalis* plasmid pAD1, donor cells require prior induction by pheromone to become fully effective in conjugating in liquid medium, whereas conjugation in filter matings requires no induction (Clewell, 1993). Plating of uninduced donor-recipient mixtures yielded a conjugation frequency of 10^{-5} the normal frequency (Ike & Clewell, 1984), which argues that pair formation by random placement of *E. faecalis* cells on the plate surface was negligible. Our results with *S. acidocaldarius* thus suggested that the cell-cell associations presumed to initiate conjugation formed before or during plating.

In addition to the quantitative considerations above, we were able to experimentally support the hypothesis that conjugation occurs in liquid suspension, using an MPN (serial dilution) method. Washed cells of Pyr⁻ parental strains were combined in selective liquid medium at an initial cell density of about 3×10^7 cells ml⁻¹. After brief (30–60 min) incubation at 22 °C, five such mixed suspensions were diluted to extinction in selective medium to enumerate Pyr⁺ recombinants, and in non-selective medium to enumerate viable cells. Controls included (i) otherwise identical dilution series of single strains only, and (ii) aliquots of the dilution series plated on solid

Table 3. Assays of genetic exchange in liquid medium

Washed cell suspensions were kept separate or combined as indicated; enumeration was by dilution in microdilution dishes (MPN) or by plating. The frequency of Pyr⁺ clones is expressed per 10^6 c.f.u.

Trial	Strain(s) tested	Pyr ⁺ frequency (MPN)	Pyr ⁺ frequency (plating)
1*	DG29	2.300	5.00
	DG38	0.670	<0.67
	DG29 × DG38	63.000	66.00
2	DG29	141.000†	0.70
	DG38	<0.025	<0.60
	DG29 × DG38	400.000‡	715.00‡

* In this trial, c.f.u. were estimated from absorbance readings of washed suspensions.

† The MPN method overestimated reversion frequency of strain DG29 due to residual growth in selective media, which is characteristic of the *pyrD1* allele. A more accurate estimate of reversion is given by plating.

‡ Mean of duplicate determinations.

media. The results (Table 3) show that enumeration by dilution in liquid media yielded recombinant frequencies comparable to those observed using plates. Growth at the end points of dilutions in selective medium could not be attributed to cross-feeding between the mutants; over 70% of the c.f.u. from these wells were prototrophs, compared with no (i.e. <2%) prototrophs recovered from the non-selective dilution series. Furthermore, similar results (not shown) were obtained with pairwise combinations of the three *pyrF* strains MR27, 39 and 41, which cannot cross-feed. The results of Table 3 thus demonstrate that efficient marker transfer does not require plating of *S. acidocaldarius*.

Fig. 3. Efficiency of genetic exchange as a function of parental cell ratio. Non-agitated liquid suspensions (0.2 ml containing 2×10^6 cells total) were mated as described in Methods; the proportion of either parental strain was varied from 0 to 100%. Total c.f.u. after incubation, as well as corrections for reversion, were calculated from the 0/100% (pure parental) suspensions in each series. ○, DG29 × DG38; □, MR27 × MR41; △, MR39 × MR41.

Additional tests of a conjugational mechanism

We investigated whether the apparent initiation of genetic exchange in liquid suspension exhibited two properties expected of conjugation, i.e. (i) requirement for contacts between cells of different genotype, and (ii) insensitivity to extracellular DNase. In the first series of tests, we conducted mating assays in liquid, keeping the total number of cells constant but varying the ratio of parental strains up to 200-fold. The resulting recombinant frequencies were distributed in roughly symmetrical manner about maxima occurring near a ratio of 1 (Fig. 3). This pattern is expected when at least one cell of each parental type is required to form a recombinant (Maloy *et al.*, 1994).

To test the effect of DNase, we took advantage of the initiation of genetic exchange in *S. di* suspensions at 22 °C. DNase I was fully active under these conditions, as indicated by spectrophotometric assay, but exerted no inhibitory effect on genetic exchange (Table 4). These results provide additional evidence that chromosomal marker exchange in *S. acidocaldarius* proceeds via conjugation (Grogan, 1996a).

Tests for conditions that prevent genetic exchange

In further attempts to control, and thereby experimentally analyse, the initiation of conjugation, various chemical agents were tested for their effect on marker exchange initiated in liquid suspension at 22 °C. The low-specificity protease from *Streptomyces griseus* ('Pronase') was tested because proteases destroy the

Table 4. Effect of hydrolytic enzymes on efficiency of genetic exchange

Mixed suspensions (5×10^7 cells of each parental strain) were incubated for 30 min at 22 °C in glass tubes before enumeration of c.f.u. and Pyr⁺ recombinants.

Treatment	Recombinant frequency (Pyr ⁺ per 10 ⁶ c.f.u.)	
	Trial 1	Trial 2
DNase*		
No additions	41, 58	
Serum albumin	38, 44	41
DNase I	93, 90	80
Pronase†		
Cells pre-mixed, no additions	5	59
Cells post-mixed, no additions	38	60
Cells pre-mixed, 1% (w/v) Pronase	343	155
1% (w/v) Pronase, cells post-mixed	108	138
Cells pre-mixed, 2% (w/v) Pronase	83	149
2% (w/v) Pronase, cells post-mixed	84	456

*DNase or bovine serum albumin was added to a final concentration of 1% (w/v) before suspensions were combined.

†'Pre-mixed' indicates that suspensions were combined before Pronase treatment, whereas 'post-mixed' suspensions were first treated separately with Pronase before being combined. Pronase treatment of cells was for 2 h at 40 °C in both cases.

cell-surface determinants of high-affinity conjugation in bacteria (Clewell, 1985). In intact *S. acidocaldarius* cells, Pronase digests externally exposed membrane glycoproteins but does not detectably affect the S-layer subunits (Grogan, 1996b). Predigestion of parental cells with Pronase did not interfere with genetic exchange under our conditions (Table 4). Other additives, primarily salts, buffers at various pH values and polymers, also failed to reproducibly prevent the initiation of genetic exchange (Table 5).

DISCUSSION

The exchange and recombination of auxotrophic markers among *S. acidocaldarius* strains is, to our knowledge, only the second example of chromosomal DNA transfer so far identified among archaea, and one of only three examples of archaeal conjugation of any type. In contrast to the mating phenomenon of *H. volcanii*, which appears to require the formation and physical stabilization of cell-cell contacts on solid supports (Mevarech & Werczberger, 1985; Rosenshine & Mevarech, 1991), exchange of chromosomal markers in *S. acidocaldarius* seemed to initiate quickly in liquid media. This was indicated by the efficient recovery of recombinants from dilute suspensions mixed at 22 °C and rapidly plated, and by the detection of recombinants

Table 5. Efficiency of genetic exchange in the presence of additives

Cells of the parental strains were suspended in Sdil buffer supplemented as indicated ('H₂O' cells were washed and resuspended in distilled water) before being combined at 22 °C. After 20 min, recombinants and c.f.u. were enumerated; recombinant frequency values are the means of duplicate determinations. EDTA (10 mM) was also tested and found to be unable to prevent recombination, but its effect on subsequent growth interfered with the quantitative assay of genetic exchange.

Mating conditions	Pyr ⁺ per 10 ⁶ c.f.u. (% of control)		
	Trial 1	Trial 2	Trial 3
Standard	28 (100)	131 (100)	25 (100)
H ₂ O only			10 (40)
0.1 M sodium acetate			
pH 4.8	41 (146)		
0.02 M potassium phosphate/malate			
pH 2.6	<0.5 (<2)	63 (48)	22 (88)
pH 3.1		198 (151)	9 (36)
pH 3.6		151 (115)	23 (92)
pH 5.2	35 (125)		
0.2 M KCl	<0.1 (<4)	52 (40)	
0.2 M MgSO ₄	120 (429)		
10% (v/v) PEG 400	26 (93)		
0.1% gellan gum		113 (86)	

in dilute cell suspensions which were never plated. Formation of recombinants was not prevented or reversed by any of several chemical agents, including DNase I and broad-specificity protease. We have thus failed so far to identify a practical method of blocking genetic exchange once two cell suspensions have been combined. Although this failure provides evidence of a specific and effective mode of cell pairing, it also limits our ability to dissect the mechanism of transfer in the way in which bacterial conjugation has been analysed (Clark & Warren, 1979; Willets & Skurray, 1980; Macrina & Archer, 1993; Clewell, 1993).

In their study of *Sulfolobus* plasmid pNOB8, Schleper *et al.* (1995a) used genomic DNA analysis to demonstrate cell-cell transfer in liquid culture, fluorescence microscopy to detect rapid formation of mixed cell aggregates of donor and recipient strains, and scanning electron microscopy to suggest the formation of cytoplasmic bridges within these aggregates. Our data seem generally consistent with a similar mechanism for *S. acidocaldarius* chromosomal transfer, but the genetic assay is much more sensitive. As a result, only about 0.03% of the cells in an *S. acidocaldarius* mixture must conjugate to yield the recombinant frequencies that we observed. This minimum is about 1/100th of the frequency of cell pairs (4%) found in growing *Sulfolobus* cultures, the majority of which probably represent cell division intermediates (Poplawski & Bernander, 1997). Our own preliminary microscopy of washed DG29 and DG38 cells estimates pairs and aggregates to represent 7% of particles in suspension, and they are not more abundant in mixtures (data not shown). In contrast to pNOB8

transfer, the intrinsic genetic exchange mechanism of *S. acidocaldarius* may thus prove impractical to analyse microscopically.

Whatever the actual frequency of pairing, the molecular architecture of *H. volcanii* and *Sulfolobus* cell envelopes implies that conjugation involves interaction between outer faces of the S-layers (Baumeister & Lembecke, 1992), which seems consistent with experimental observations. Bacterial S-layers may mediate specific cell-cell attachment, as suggested by the spontaneous pairing of *Deinococcus* S-layers in perfect register at their extracellular faces (Baumeister & Hegerl, 1986). Similarly, intact S-layer sacculi purified from *S. acidocaldarius* by detergent extraction spontaneously aggregate in suspension. This clumping, which can interfere with the use of S-layer fragments as nanometre-scale surface-patterning templates (see Douglas *et al.*, 1992), can only be avoided through particular growth and purification conditions (J. Moore, personal communication). Our observation that 2 h Pronase treatment did not decrease fertility of *S. acidocaldarius* may also be significant, since S-layer subunits are the only externally exposed cell envelope proteins of *S. acidocaldarius* known to be completely resistant to Pronase digestion (Grogan, 1996b).

Taken together, our results are consistent with the hypothesis that *S. acidocaldarius* cultures contain a proportion (apparently small) of cells able to rapidly form cell-cell associations in dilute liquid suspension, leading eventually to chromosomal exchange and homologous recombination. This hypothesis raises a series of mechanistic questions, and a variety of physiological

parameters, which have yet to be evaluated, apparently affect the efficiency of genetic exchange. The recombinant frequency for a pair of strains under defined conditions varied considerably among different trials (e.g. Fig. 1; Table 5). As a function of growth phase, we have found the fertility of cultures to be highest at the lowest cell densities, gradually declining to about 10% of its original value in stationary phase (data not shown). In other experiments, parental cells were incubated separately at 75 °C on solid non-nutrient medium before being combined. Under these conditions, fertility declined several-fold over a period of 30 h. This decline, coupled with a concomitant increase in recombinants from mixtures similarly incubated (data not shown), helps to explain the fact that, in prior studies, control plates yielded significantly lower recombinant frequencies than mated mixtures (Grogan, 1996a).

The frequency and apparently robust nature of chromosomal marker exchange in *S. acidocaldarius* has implications for gene flow and evolutionary dynamics of *Sulfolobus* populations in geothermal habitats. The range of conditions tested in the present study was constrained by our focus on straightforward laboratory techniques, but it suggests that the exchange of chromosomal genes can be efficient and does not have requirements that preclude it in natural habitats of *Sulfolobus*. [A similar conclusion could be drawn for transfer of pNOB8 (Schleper *et al.*, 1995a).] The frequencies of 10^{-5} – 10^{-4} Pyr⁺ recombinants per c.f.u. typically observed in the present study greatly exceed the rate of spontaneous mutation in *S. acidocaldarius*, which was recently estimated to be $1\text{--}3 \times 10^{-7}$ mutational events per cell division for the *pyrE* and *pyrF* genes (Jacobs & Grogan, 1997). Although marker exchange is probably less frequent in nature than under our optimized laboratory conditions, recombination rates need not be this high to exert an effect on genetic properties of populations. Theoretical models predict that much lower rates of recombination can confer sexual character upon suitable populations of prokaryotes (Cohan, 1994; Maynard Smith, 1994). It is interesting to speculate that the enhanced genetic diversity due to out-crossing may be sufficiently advantageous so as to maintain chromosomal exchange in natural populations of *S. acidocaldarius*. [It must be borne in mind, however, that demonstration of spontaneous genetic exchange under laboratory conditions does not guarantee its predominance in nature; bacteria known to exchange genes in laboratory experiments have apparently clonal population structures in nature (Maynard Smith *et al.*, 1993).] Alternatively, conjugation may primarily benefit *S. acidocaldarius* as a mechanism of DNA repair, the genetic consequences of which, though beneficial, are indirect and secondary (Bernstein *et al.*, 1985).

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