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Genetic Transformation of Obligately Chemolithotrophic Thiobacilli

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Genetic transformation of *Thiobacaillus thioparus* auxotrophs to prototrophy was obtained at frequencies of up to 10^{-2} when proliferating cell populations were exposed to chromosomal DNA from a nutritionally independent strain of the same bacterium. The rate at which transformation occurred depended on recipient growth rate and could be drastically reduced by depriving otherwise competent cells of either nitrogen or exogenous energy substrate. Interspecies marker transfer was also shown among several obligately chemolithotrophic members of the genus.

Lack of defined mutants has hitherto limited genetic examination of thiobacilli to the comparison of their DNA base composition (14). The recent development of procedures for obtaining the requisite mutants from both obligately and facultatively chemolithotrophic members of the genus (10, 17) has made it possible to begin searching for suitable methods of effecting interspecies marker exchanges. We here report on the development of one such system, the high-frequency genetic transformation of *Thiobacillus thioparus* and a related species with purified DNA of homologous and heterologous origin.

MATERIALS AND METHODS

Bacterial strains. Strain SN is a streptomycin and nalidixic acid resistant mutant of *T. thioparus* ATCC 8158. Strains SNL, SNP, and SNT are, respectively, leucine, proline, and tryptophan auxotrophs of strain SN. In SNL, the basis for leucine deficiency is known to be the lack of β-isopropylmaleic acid isomerase function (17). The procedures used to obtain and characterize these mutants have been described elsewhere (17), as have those employed in the isolation of similar derivatives of *Thiobacillus* sp. strain A2 (10). Extension of the appropriate procedures to other thiobacilli made available nalidixate-resistant (Nal^r) mutants of *Thiobacillus neapolitanus* C (4), *Thiobacillus* sp. strain A2 (21), and *Thiobacillus* sp. strain Y. The latter bacterium, an obligately chemolithotrophic auxotroph obtained from river mud in Israel, exhibits morphological, nutritional, and physiological properties similar to those of *T. thioparus* and *T. neapolitanus* C. It can, however, be distinguished from the former species by its inability to develop on thiocyanate in liquid culture, and from the latter by a similar lack of growth on tetrathionate (22).

Media and culture conditions. Liquid culture of obligately chemolithotrophic strains was made on ATM medium (pH 7.1) containing 10 mM thiosulfate or on the same medium supplemented with 50 μg of required amino acid ml^{-1} (17). Colony formation was on ATM-thiosulfate agar plus (or minus) 50 μg of required amino acids, nalidixic acid, or both ml^{-1}. Modified Taylor and Hoare medium (21) was used to cultivate strain A2N of *Thiobacillus* sp. strain A2, either chemautotrophically on 20 mM thiosulfate or chemoheterotrophically on 20 mM succinate (10). When required, nalidixic acid was present at 50 μg ml^{-1}. Culture of *Thiobacillus* sp. strain A2 was also carried out on nutrient broth in the presence and absence of 0.2% glucose.

Recipients for transformation experiments were routinely harvested from early exponential-phase cultures at cell densities below 2×10^7 CFU ml^{-1} as already detailed (17). Also given elsewhere are the procedures used for growing and harvesting larger culture volumes for purposes of DNA isolation (17).

Isolation of DNA. Late-exponential-phase cultures were harvested, suspended in 10 mM EDTA–150 mM NaCl (pH 8.0), lysed with sodium dodecyl sulfate, and deproteinized by chloroform-isoamyl alcohol extractions followed by ethanol precipitation, as already described (18). In some cases, further digestion of crude DNA with pancreatic RNase I was carried out by the method of Marmur (11). Crude or RNase-digested fibers of DNA were sterilized by immersion in 70% ethanol for 48 h and then dissolved in sterile SSC (1.5 mM NaCl, 0.15 mM trisodium citrate, pH 7.0) buffer. Preparations were stored frozen or at 4°C over chloroform and quantitated by the diphenylamine procedure of Burton (1). About 1 to 2 mg of DNA was obtained per g of cells (wet weight) processed.

Size reduction of native DNA fragments by mechanical shearing. RNase-digested DNA at a concentration of 250 μg ml^{-1} in SSC buffer was subjected to 10 passages through a syringe equipped with 25-gauge needle.

Estimation of DNA molecular weight. Sedimentation velocity runs in the Spinco E analytical ultracentrifuge were carried out at 20°C. Samples to be tested contained 25 μg of RNase-digested DNA ml^{-1} in double-

strength (2×) SSC buffer or alkaline SSC (1 volume of 2× SSC plus 1 volume of 0.3 N NaOH). Apparent s_{20} values were calculated from the midpoint of the sedimenting boundary and used without correction to estimate mean particle weights according to the empirical equations of Studier (20) for native and alkali-denatured DNA: native DNA, $s_{20} = 0.0882\ M^{0.346}$; alkaline DNA, $s_{20} = 0.0528\ M^{0.400}$.

Size resolution of native DNA by preparative ultracentrifugation through neutral sucrose gradients. Gradients of from 5 to 20% (wt/wt) sucrose were prepared in nitrocellulose tubes over an 0.5-ml cushion of 70% sucrose. All solutions contained 10 mM Tris and 1 mM EDTA at pH 7.5. Gradients were overlayered with 0.5-ml of sample containing 900 μg of DNA in 1/100 SSC buffer to give a total volume of 11.5 ml and then centrifuged for 2 h at 38,000 rpm and 18°C in the SW40 rotor of the Spinco L65 centrifuge. Fractions of 0.5 ml each were collected by drop counting from bottom to top of the gradient tube, diluted with 0.8 ml of 1/100 SSC buffer, and analyzed for their DNA content by the method of Burton (1).

RESULTS

Transformation of *T. thioparus* SNL from leucine auxotrophy to prototrophy. The frequency of leucine-independent (Leu^+) revertants in strain SNL populations was previously shown to be 5×10^{-8} (17). Nevertheless, more than 0.05% of the exponential-phase strain SNL cells plated on ATM agar without L-leucine rapidly formed colonies when DNA from *T. thioparus* SN was included in the plating mixture (Table 1). Since contamination of the DNA preparation with live strain SN (i.e., Leu^+) cells could be ruled out, the transformation of SNL cells from Leu^- to Leu^+ must account for the colony appearance on selective plates. This phenotype transformation was inhibited by DNase, unaffected by RNase or pronase, and not elicited by homologous DNA in place of DNA from the prototrophic strain SN donor (Table 1). Accordingly, DNA-mediated transfer of the functional β-isopropylmaleic acid isomerase gene(s) from strain SN to SNL was suggested.

Effect of donor DNA size and state on strain SNL transformation. The mean particle weight estimates obtained by sedimentation analysis of RNase-digested DNA from *T. thioparus* SN in neutral and alkaline environments were, respectively, 4.5×10^7 and 2.8×10^6. Thus, although fairly large on the average, the native genome fragments isolated from this bacterium by routine means carried numerous single-strand breaks. Based on preparative sedimentation through a neutral sucrose gradient, the native DNA preparation in question was also characterized by a high degree of particle size heterogeneity (data not shown). Biological activity was, however, found, with DNA structures representing almost the entire available size range.

Mechanical shearing reduced the average particle weight of the DNA fragments from 4.5×10^7 to 1.1×10^6 and resulted in a 30-fold drop in specific transforming activity relative to unsheared material (Fig. 1A). Similar results were obtained by others (2, 8) for *Pneumococcus*. Since the information-carrying strands in sheared duplexes were shorter on average than the single strands present in alkali-denatured DNA, it cannot be argued that strand length is the reason for biological inactivity in the latter case (Fig. 1A).

The number of Leu^+ transformants appearing on selective plates containing a fixed number of strain SNL recipients was linearly related to the quantity of either sheared or unsheared double-stranded DNA added to the plating mixture (Fig. 1A). However, the proportionality constant relating DNA dose to transformant frequency was significantly different at low (<1 μg per plate) and high DNA inputs (Fig. 1B). Furthermore, the same biphasic dose-response curve, with a break point at about 1 μg of DNA per plate, was obtained when the total number of strain SNL recipients was shifted from 5×10^4 (Fig. 1B)

TABLE 1. Transformation of *T. thioparus* SNL to leucine independence

Composition of plating mixture	CFU plated[a] (×10⁶)	No. of colonies formed on selective medium[b]
SNL cells[c]	2.2	0
SNL cells + SN DNA[d]	1.8	>1,000
SN DNA	0	0
SNL cells + DNA + DNase[e]	2.0	0
SNL cells + SN DNA + RNase	1.7	>1,000
SNL cells + SN DNA + pronase	1.8	>1,000
SNL cells + SNL DNA	2.0	0

[a] Determined by serial dilution and plating on thiosulfate agar supplemented with 50 μg L-leucine ml^{-1}.

[b] Colonies arising after 3 days at 30°C on thiosulfate agar lacking L-leucine.

[c] An experimental culture of *T. thioparus* SNL at 10^7 CFU ml^{-1} was harvested, and the cells were washed free of L-leucine and resuspended in 0.5 volume of double-strength ATM medium containing thiosulfate but not L-leucine.

[d] Crude DNA from strain SN or SNL was dissolved at 12 μg ml^{-1} in 1/100 SSC and mixed with cells (or cell-free medium) in a ratio of 1:1 (vol/vol).

[e] Crude DNA was exposed to 10 μg of pancreatic DNase I ml^{-1} or RNase in 1/100 SSC containing 1 mM $MgCl_2$ for 15 min at 37°C. Self-digested pronase (9) was added to the DNA at 1 mg ml^{-1} under otherwise identical conditions.

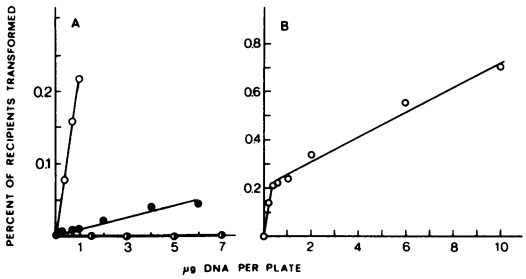

FIG. 1. Relation of transformation activity to (A) donor DNA size and state and (B) donor DNA dose. Mixtures of RNase-digested DNA and exponential-phase cells from *T. thioparus* SN were arranged to give the indicated quantities of DNA and 5×10^4 cells on ATM plates lacking L-leucine. Colonies were counted after 3 days at 30°C. Symbols: ○, unsheared DNA; ●, sheared DNA; ◑, alkali-denatured DNA.

down to 5×10^3 or up to 5×10^5. Thus, a certain fraction of the cells in any given population might be in a physiological state which renders them competent to respond to relatively low doses of DNA, whereas the remainder are only capable of undergoing transformation in the presence of higher amounts of DNA. Alternative explanations for the same phenomenon in *Pneumococcus* have been discussed by Porter and Guild (16).

Recipient growth as a requisite for transformation. Strain SNL (Leu$^-$) cells prepared from early-exponential-phase cultures proved to be the most competent recipients in the direct plating assay with donor DNA from strain SN. Direct plating of exponential-phase strain SNT (Trp$^-$) and SNP (Pro$^-$) cells on unsupplemented agar with strain SN DNA did not, on the other hand, result in the appearance of prototrophic transformants. Since, of these *T. thioparus* mutants, only strain SNL retains the residual ability to multiply in the absence of its required amino acid (17), its seemingly unique capacity to undergo transformation might well reflect a need for continued recipient multiplication during exposure to donor DNA. In agreement with this idea was the outcome of an experiment in which exponential-phase strain SNL cells were exposed to strain SN DNA for up to 5 h on aerated medium lacking L-leucine or both L-leucine and a general nitrogen source (i.e., NH_4Cl). With only the amino acid lacking there was a discernible, albeit small, rise in the viable cell titer of the culture over the course of the experiment, and an even sharper rise in Leu$^+$ cell frequency (Fig. 2A). By contrast, nitrogen-starved cells remained essentially stationary in both number and Leu$^+$ frequency (Fig. 2B). It might also be noted that the rise in Leu$^+$ cell frequency with nitrogen present (Fig. 2A) must reflect the production of new transformants over time. Given the relatively slow rate of reproduction (0.3 generations h^{-1}) of wild-type cells (17), multiplication of early transformants could hardly have made any significant contribution to the end result.

General transformation of thiobacilli. Multiplying strain SNT and SNP recipients in liquid culture were as readily transformed to prototrophy by strain SN DNA as were strain SNL recipients (Table 2). High-frequency transformation of proliferating *Thiobacillus* sp. strain Y cells from nalidixate sensitivity to resistance was also obtained in the presence of DNA from strain YN (Table 2). A corresponding transformation of *T. neapolitanus* C to nalidixate resistance by strain CN DNA was not, however, observed (Table 2). Also negative were efforts to obtain drug-resistant transformants of *Thiobacillus* sp. strain A2 by exposure to DNA from a suitably marked strain of the same facultative chemolithotroph (Table 2). Failure to obtain transformants in the latter two cases included efforts with recipients prepared in different ways from cultures in different stages of growth and, for *Thiobacillus* sp. strain A2, on a range of growth media. Repetition with different DNA preparations did not alter the negative outcome. It might also be noted that the mean size of double-stranded donor DNA fragments ap-

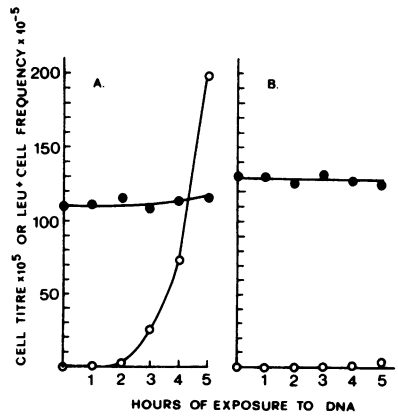

FIG. 2. Transformation kinetics of strain SNL recipients exposed to strain SN DNA in leucine-free (A) and nitrogen-depleted (B) media under otherwise growth-permissive conditions. Samples of 0.2 ml each were removed at the indicated times, added to 0.2 ml of ATM medium containing 4 μg of DNase, and incubated at 30°C for 15 min. Samples of 0.1 ml from each DNase digestion mixture were then scored for Leu$^+$ transformants on ATM-thiosulfate agar and, after suitable dilution, for total CFU on leucine-supplemented agar. Symbols: ●, CFU; ○, transformants.

TABLE 2. Homologous transformation of *Thiobacillus* species

Recipient strain	Donor strain	Donor DNA state[a]	Selected phenotype[b]	Recipients transformed[c] (%)
T. thioparus SNT	SN	Crude, native	Trp^+	0.16
		DNase-digested		0
T. thioparus SNP	SN	Crude, native	Pro^+	0.28
		DNase-digested		0
Thiobacillus sp. strain Y	YN	Crude, native	Nal^r	0.17
		DNase-digested		0.01
		RNase-digested		0.13
T. neapolitanus C	CN	Crude, native	Nal^r	0
Thiobacillus sp. strain A2	A2N	Crude, native	Nal^r	0

[a] DNA was added to exponential cultures containing 2×10^6 to 5×10^6 CFU ml^{-1}. Amounts of DNA added were 10 μg of SN or YN, 45 μg of CN, and 80 μg of A2N material per ml. Predigestion of nucleic acids was as described in Table 1, footnote *e*.

[b] Trp^+, Pro^+, and Nal^r refer to tryptophan independence, proline independence, and nalidixate resistance, respectively.

[c] Exposure to DNA under conditions of full recipient growth was for 4 h in the case of SNT and SNP, 18 h in the case of *Thiobacillus* sp. strain Y, and 24 h in the cases of *T. neapolitanus* C and *Thiobacillus* sp. strain A2. Cell-DNA interaction was stopped with DNase as described in the legend to Fig. 2.

proached (or exceeded) 10^7 daltons in each case. No attempts were made to chemically or enzymatically alter the surface envelopes of transformation-negative strains (7).

Relation of growth and transformation kinetics. Slow uptake of amino acids by *T. thioparus* strains leads to a situation in which the rate of growth of auxotrophs can be easily controlled by the amount of required amino acid supplied (17). Accordingly, it becomes possible to relate recipient growth and transformation kinetics under essentially constant conditions by adding DNA to a series of young cultures containing different concentrations of the pertinent amino acid. When cells in such a series of exponential-phase strain SNL cultures were exposed for 1 h to strain SN DNA with continued aeration, treated with DNase, washed free of L-leucine, and plated on thiosulfate agar with and without L-leucine, Leu^+ cell frequency increased with growth rate (Fig. 3). The most competent strain SNL recipients were those dividing at the maximal rate under optimal conditions when exposed to DNA.

The time required for a strain SNL population actively growing in the presence of strain SN DNA on excess (50 μg ml^{-1}) L-leucine to even approach saturation with respect to Leu^+ cell frequency was approximately 4 h (Fig. 4A). Moreover, since neither Leu^+ or Leu^- cell types had a growth advantage under these conditions, any rise in Leu^+ frequency per unit of time must represent the occurrence of new transformation events during the time period in question. Removal of the energy substrate (i.e., thiosulfate) from the medium prevented otherwise competent recipients from undergoing transformation (Fig. 4B). Thus, the *T. thioparus* transformation system appears to be characterized by intrinsically slow kinetics and stringent requirements for ongoing growth and energy substrate metabolism. On the other hand, the

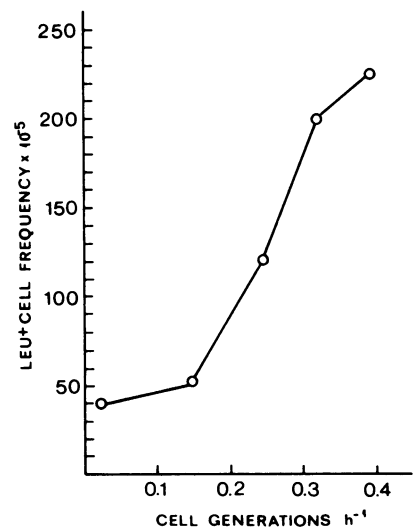

FIG. 3. Effect of recipient growth rate on transformation kinetics. To a series of cultures containing between 5×10^6 and 8×10^6, *T. thioparus* SNL cells ml^{-1} and enough (2 to 50 μg ml^{-1}) L-leucine to support the indicated rates of growth was added 6 μg of crude DNA ml^{-1} from *T. thioparus* SN. Each culture was aerated at 30°C for 1 h, then treated with DNase, and sampled for total and Leu^+ cell types on leucine-supplemented and selective agars as described in the legend to Fig. 2.

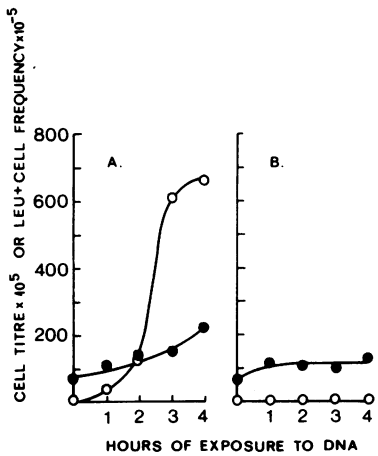

FIG. 4. Transformation kinetics of rapidly dividing (A) and substrate-deprived (B) strain SNL recipients in liquid medium. An early exponential-phase culture of *T. thioparus* SNL cells was harvested at room temperature, resuspended in ATM medium containing 10 μg of DNA ml^{-1} from *T. thioparus* SN, and divided into two parts; one part received 10 mM thiosulfate. Both were then aerated at 30°C for 4 h. Samples were assayed for total and Leu^+ cell titer at 1-h intervals throughout. Symbols: ○, Leu^+ transformant frequency; ●, CFU.

system is efficient in the percentage of recipients transformed (Fig. 4A). Thus, between 0.1 and 1% of the available recipients were routinely recovered as Leu^+ transformants in repetitive experiments with different cell and DNA preparations.

Interspecies marker transfer. Prolonged (4 to 24 h) interaction between actively dividing cells of *T. thioparus* SNP and DNA from either *Thiobacillus* sp. strain YN or *T. neapolitanus* CN in liquid culture led to the appearance of Pro^+ transformants (Table 3). The transformation frequencies observed in these heterologous systems were, respectively, one-third and one-twentieth that obtained with a homologous (i.e., strain SNP cells plus strain SN DNA) mixture (Table 3). Similarly, Nal^r transformants of *Thiobacillus* sp. strain Y arose one-third and one-tenth as frequently, respectively, with DNA from strains CN and SN compared with those from strain YN DNA (Table 3). These findings clearly showed that *T. thioparus*, *T. neapolitanus* C, and *Thiobacillus* sp. strain Y are genetically related but distinct microorganisms. Furthermore, the fact that strain CN DNA showed biological activity with *T. thioparus* and *Thiobacillus* sp. strain Y (Table 3) but not with *T. neapolitanus* C (Table 2) appears to be due to the lack of recipient competence. Since DNA from *Thiobacillus* sp. strain A2N failed to elicit even homologous transformation (Table 2), we could not conclude with equal certainty that its corresponding inability to effect genetic marker transfer in other members of the genus (Table 3) derived from phylogenetic considerations. Nevertheless, since physical examination of strain A2N DNA revealed the presence of large ($>10^7$ daltons) double-stranded structures, there was no a priori reason to suspect a total lack of transforming potential. Therefore, the repetitive failure of strain A2N DNA to transform either *T. thioparus* or *Thiobacillus* sp. strain Y recipients (Table 3) probably implies a rather distant relationship between *Thiobacillus* sp. strain A2 and the latter species.

DISCUSSION

The simplest explanation for transforming DNA size heterogeneity was random origin from the donor strain chromosome. Since, in addition, the smallest active fragments were of the size order of single genes, transforming DNA from *T. thioparus* appeared not to differ in any fundamental way from analogous material from other bacteria (2, 8, 16).

Removal of the energy substrate led to immediate loss of transformation ability by otherwise competent recipients. This demonstration of the energetic nature of the transformation process probably reflects a lack of endogenous energy reserves in *T. thioparus* (15, 22). Stringent blocking of substrate catabolism by inhibitors of energy production (e.g., cyanide) is required to demonstrate the same effect in chemoorganotrophic heterotrophs.

TABLE 3. Heterologous transformation of *Thiobacillus* species

Recipient strain	DNA donor strain	Selected marker	Transformants[a] (%)
T. thioparus SNP	SN	Pro^+	0.40
	YN		0.14
	CN		0.02
	A2N		0
Thiobacillus sp. strain Y	YN	Nal^r	0.44
	CN		0.15
	SN		0.05
	A2N		0

[a] Each value represents the average of three independent trials carried out in the presence of 40 μg of DNA ml^{-1} in the series with SNP recipients, and 80 μg ml^{-1} with Y recipients. Reaction in liquid exponential cultures was for 18 to 24 h. It was established in every case that prior treatment of the DNA with DNase abolished transformation, whereas digestion with RNase had little or no effect.

The development of competence for transformation during exponential-phase growth has been reported in several cases (3, 19). Nevertheless, the correlation of transformation and recipient division rates is, as far as we are aware, a peculiarity of the obligately chemolithotrophic bacteria in question. Also unusual were the relatively slow transformation kinetics observed even under what appeared to be ideal growth conditions. Since DNA uptake was controlled by DNase addition in the kinetic experiments, the slow overall rate of transformation observed almost certainly reflected a correspondingly slow rate of DNA adherence to or passage through cell surface barriers. Thus, although direct measurement of uptake rates with labeled DNA has not yet been undertaken, it is difficult to avoid comparing the relatively slow transformation rates observed here with our previous demonstration of slow amino acid transport in *T. thioparus* (17, 18) and with the generally poor response of obligately chemolithotrophic autotrophs to organic molecules of all types (5, 12, 13).

In contrast to rate, transformation frequencies compared well with those reported for other bacterial systems (6, 8). A practical consequence of the high-frequency response obtained is the construction of multiple-factor strains from different mutants. Thus, transformation and selection for the transformant phenotype have already been used in *T. thioparus* to transfer amino acid biosynthesis, motility, and transport markers from strain to strain (18). The construction of multiple-factor strains by heterologous transformation should also be possible. Finally, the availability of a gene transfer system adds a whole new dimension to the classification of thiobacilli along natural lines.

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