Isolation and Characterization of a Transformation Deficient Mutant of *Streptococcus pneumoniae* that is Defective in the Production of an Activator

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Transformation deficient mutants were isolated by nitrosoguanidine mutagenesis. A mutant Tra-12 was defective in the spontaneous development of competence for genetic transformation during culture growth, and showed no activator (competence inducing factor) activity inside or outside of the cells throughout growth. However, Tra-12 exhibited normal competence after the addition of an activator extracted from the wild type strain. This mutant needed a much larger quantity of the activator to develop a saturating level of competence and the induced competence disappeared more rapidly than in the wild type strain.

In *Streptococcus pneumoniae*, a hormone-like macromolecular cell product called an activator plays a crucial role in the development of competence for genetic transformation. It serves as a population density monitor, and is present only in competent cells.^{1~4)} The interaction of the activator and incompetent cells leads to the temporary synthesis of a small set of proteins, including the activator itself,^{3,5)} and induces changes in the cell surface that are specific to competent cells.^{6,7)} Specific DNA binding activity in competent cells appears a few minutes after the addition of the activator.⁸⁾ A nick is introduced in the double-stranded DNA bound at the surface, and then a single-stranded fragment is introduced into the cell with the concomitant degradation of the complementary strand.^{8,9)} The fragment forms a complex with one of the competence-induced proteins (eclipse complex), and then the fragment is inserted into the chromosome at a homologous site.^{10,11)} This series of successive complex reactions is triggered by the activator, which appears to be a key protein in genetic

transformation. However, it is not known how the signal for attachment of the activator to the cell surface is transmitted to the cell, how protein synthesis is induced, or how the population density is monitored in the development of competence during culture growth. Mutants that are defective in the development of competence would be useful for answering these questions. So far several transformation deficient mutants have been found,^{12~14)} but few mutants that are defective in the process of development of competence have been obtained. We isolated a mutant that appeared to be defective in the production of the activator.

MATERIALS AND METHODS

Bacterial strains and media. A derivative (R6) of wild type *Streptococcus pneumoniae* strain R36A¹⁵⁾ was used for the mutagenesis and as a control in the screening test. Streptomycin resistance marker DNA was extracted from the Rst7 strain. A streptomycin resistant (Str^r) and thymidine dependent strain, Tvs1, was used for preparation of ³H-labeled DNA. The R6-ery strain, which is erythromycin resistant (Ery^r), was prepared from the R6 strain by

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nitrosoguanidine mutagenesis in our laboratory, and used to detect activator activity in the medium of a competence-induced culture. The compositions of the growth medium (C medium) and competence medium (CpH8+alb medium) were given previously.³⁾

Transformation assay. The following experimental procedures were described elsewhere; preparation of the activator⁴⁾ and transforming DNA,¹⁶⁾ and determination of viable counts and transformants on agar plates.¹⁷⁾ The preparation and storage of standardized competent cells and incompetent cells were reported previously,¹⁷⁾ and modified as follows. Competent cells grown in CpH8+alb medium (competence medium) and incompetent cells grown in low pH (pH 6.8) C medium, in which the cells remain physiologically incompetent, were harvested at 1×10^8 cells/ml and then stored at -80°C . For rendering incompetent cells competent with the activator (activation), 0.1 ml of the activator was added to incompetent cells (1×10^7 cells/ml) in 0.9 ml of competence medium, followed by incubation at 30°C for 20 min. Then 1 μg of trypsin was added to prevent further activation of the cells. To examine the transformability of competence-induced cells, 0.1 ml of donor DNA (1 μg) was added. After 20 min at 30°C , 2 ml of C medium containing 3 μg pancreatic DNase I was added, and then the culture was incubated at 37°C for 100 min. Transformants were then scored on selective agar plates containing streptomycin (150 $\mu\text{g}/\text{ml}$).

Mutagenesis. A culture of R6 grown in C medium (1×10^8 cells/ml) was exposed to 20 $\mu\text{g}/\text{ml}$ of 1-methyl-3-nitrosoguanidine (NTG) for as long as it took the number of viable cells to decrease to 0.1% of the original cell counts. For segregation, the culture was diluted 10-fold in growth medium and then grown to 3×10^8 cells/ml. The cells were then stored at -80°C . This NTG treatment was repeated 10 times, and the resulting culture was used to screen for transformation deficient mutants.

Screening. NTG-treated cells were incubated in fresh medium until the cell density reached 3×10^8 cells/ml. The culture was then diluted 10^5 -fold and plated on a DNA plate of CpH8 medium containing 250 units/ml catalase, 0.08% bovine serum albumin, 0.1% yeast extract and 50 $\mu\text{g}/\text{ml}$ donor DNA (Str^r). The resulting colonies were tested for streptomycin resistance by replication. This DNA plate assay was repeated several times with streptomycin sensitive colonies, and the resulting 34 streptomycin sensitive colonies were stored at -80°C . The liquid transformation assay was performed for each strain; 1 μg of transforming DNA (Str^r) was added to 1 ml of the growing culture at the early exponential growth phase, followed by incubation for 3 hours to a cell density of 1×10^9 cells/ml. Each culture was chilled on ice and then the transformants were scored on 0.7% agar plates containing 150 $\mu\text{g}/\text{ml}$ of streptomycin.

Cellular DNA binding assay. ³H-labeled DNA carrying the Str^r determinant was prepared from the Tvs1 strain.⁸⁾ The DNA binding assay was performed as follows; frozen 2 ml samples of incompetent cells and competent cells were thawed on ice and then centrifuged. The pellets were suspended in 1 ml of competence medium. To the suspension of incompetent cells, the activator (0.1 ml) was added, followed by incubation at 30°C for 20 min to induce competence. Then ³H-labeled DNA (1 μg) was added. After incubation at 30°C for 20 min, the cell-associated radioactivity was determined by the "centrifugal assay."¹⁸⁾

RESULTS

Mutagenesis and screening

Wild type cells (R6 strain) treated with NTG were plated on DNA plates containing 50 $\mu\text{g}/\text{ml}$ of donor DNA (Str^r), and the resulting colonies were tested for streptomycin sensitivity by replication. For the two thousand streptomycin-sensitive clones obtained, the DNA plate assays were repeated. Finally, 34 streptomycin-sensitive clones were picked up as candidates for transformation-deficient mutants. Liquid transformation assays were performed for each streptomycin-sensitive clone. As a result, we obtained 8 mutants that were completely defective in the development of competence when grown in a medium favoring competence (CpH8+alb medium). Of these mutants, Tra-12N was characterized further in this study.

To obtain an isogenic mutant of Tra-12N, its chromosomal DNA was extracted and used to transform R6 cells. The transformation reaction mixture was plated on DNA plates, and after repeating the DNA plate assays, streptomycin-sensitive colonies were tested for transformability by the liquid transformation assay. The phenotype of the back-crossed mutant was found to be identical to that of the original Tra-12N mutant, and the back-crossed mutant hereafter is referred to as Tra-12. The following data were obtained for the back-crossed Tra-12 mutant.

Defective development of competence during culture growth of Tra-12

The R6 strain exhibited transient compe-

FIG. 1. Deficiency of Spontaneous Development of Competence in the Tra-12 Strain.

Wild type R6 cells (A) and mutant Tra-12 cells (B) were grown in the competence medium. At the indicated times, cell density (○), transformability (●) and activator activity extracted from the cells (□) were measured. One unit of activator activity in the cultures was defined as the activity that yielded one transformant.

fence when grown in CpH8 medium, and the activator could be extracted from these competent cells (Fig. 1A). In contrast, Tra-12 cells did not develop competence at any growth phase, and the activator could not be extracted from Tra-12 cells (Fig. 1B). Furthermore, no DNA binding activity was detected in Tra-12 cells (data not shown).

Induction of competence by the activator in the Tra-12 mutant

As shown in Fig. 2, competence could be induced in Tra-12 cells by the addition of the activator extracted from wild type cells. A few minutes after the addition of the activator, a significant increase in competence was observed. The time kinetics of induction of competence in the Tra-12 culture were quite similar to those in the wild type strain. However, Tra-12 cells required 3-fold more activator for induction of the maximum level of competence compared with the wild type strain (Fig. 3). Once this level had been attained, Tra-12 cells could bind and take up as much double-stranded DNA as the R6 strain (Table I).

FIG. 2. Time Kinetics of Induction of Competence by the Activator in Wild Type Cells and Tra-12 Cells.

Incompetent cells of wild type R6 (●) and mutant Tra-12 (○) grown in the low pH medium until $OD_{650\text{ nm}} = 0.15$ were diluted 10-fold with the competence medium, and then the activator was added to each reaction mixture. At various times, the activation was terminated by the addition of trypsin ($1\text{ }\mu\text{g/ml}$) and then donor DNA (Str^r) was added. The level of competence induced by the activator was expressed as the number of streptomycin resistant transformants scored.

FIG. 3. Effect of the Activator Concentration on Induction of Competence in Mutant Tra-12.

The experimental conditions and symbols are the same as in the legend to Fig. 2, except that various dilutions of the activator were added to incompetent cells, and that the activation was terminated 20 min after addition of the activator. Transformants of strain R6 (●) and strain Tra-12 (○) were scored, and then expressed as a function of the activator concentration.

TABLE I. DNA BINDING ACTIVITY IN COMPETENCE-INDUCED Tra-12 CELLS

Recipient ^a		Activator addition	³ H-DNA entry ^b (dpm)	Transformants ^c per ml
Strain	Grown to			
R6	Competence	—	48,500	3,330,000
	Incompetence	+	147,700	6,300,000
	Incompetence	—	200	< 5
Tra-12	Competence	—	370	< 5
	Incompetence	+	176,500	3,500,000
	Incompetence	—	200	< 5

^a Recipient cells were used in CpH8 + alb medium at a cell density of 2×10^8 cells/ml.

^b DNA entry is expressed as the radioactivity of donor ³H-DNA associated with the cells in an external pancreatic DNase I-resistant form.

^c Diluted samples were plated with streptomycin (150 µg/ml), and then transformants were scored.

Defect in activator production in Tra-12

The above finding suggested that the production of the activator is impaired in competent Tra-12 cells. To confirm this, a saturating amount of the activator was added to wild type R6 and Tra-12 incompetent cultures (at 0 min), and the activator activity in the culture fluid was monitored during the incubation (Fig. 4). At short intervals, a 0.45 ml portion of the culture was pipeted into another tube, and then 0.05 ml of R6-*ery* incompetent cells was added to the tube. After incubation at 30°C for 20 min, donor DNA (Str^r) was added. The activator activity in the fluid was estimated as the ability to induce competence in the added R6-*ery* incompetent cells; the appearance of transformants was scored on an agar plate containing streptomycin (150 µg/ml) and erythromycin (0.1 µg/ml). At zero min, the added activator activity was detected in the culture fluid of both Tra-12 and R6 cultures. In the R6 culture the activity increased until 100 min, due to the production of the activator by the competent R6 cells induced by the added activator. A substantial amount of the activator was detected at 200 min in the R6 culture. In contrast, the activator activity in the Tra-12 culture fluid did not increase, and no activity was detected at 140 min. These results suggested that the Tra-12 strain was defective in the production of the active activator.

After the addition of the activator (at

FIG. 4. Residual Activity of the Activator in the Competence-induced Tra-12 Culture.

A large amount of activator was added to an incompetent Tra-12 or R6 culture at 0 time. At various intervals, residual activator activity in the culture fluid and the competence level induced were determined. Open bars and closed bars indicate the residual activator activity of the R6 and Tra-12 cultures, respectively. The competence level was also determined in the R6 culture (○) and the Tra-12 culture (●).

0 min), the competence levels of R6 and Tra-12 were also determined. In the Tra-12 culture, the level of competence induced by the exogenous activator disappeared rapidly; the loss of competence of the Tra-12 culture occurred earlier than that in the case of R6, in proportion to the amount of the activator activity in the fluid. It was demonstrated that a high level of competence was maintained in the culture with

TABLE II. RECOVERY OF TRANSFORMABILITY OF Tra-12 ON TRANSFORMATION

Recipient cells	1st transformation			2nd transformation ^b	
	Activator	Donor DNA ^a	Ery ^r TF/ml	Donor DNA	Str ^r TF/ml
R6	—	—	nd ^c	str ^r (2 µg)	3,200,000
	—	—	nd	—	nd
Tra-12	+	ery ^r	2,400,000	str ^r (2 µg)	275
	+	ery ^r	2,400,000	str ^r (1 µg)	120
	+	ery ^r	2,400,000	—	nd
	+	ery ^r	2,400,000	calf thymus	nd
	+	calf thymus	nd	str ^r (2 µg)	nd
	+	—	nd	str ^r (2 µg)	nd
	—	ery ^r	nd	str ^r (2 µg)	nd

^a R6-ery DNA (carrying the erythromycin resistance marker) and calf thymus DNA were used as homologous and heterologous DNA, respectively.

^b To estimate the number of revertants of Tra-12 on the first transformation, the culture was regrown in the presence of DNA carrying the streptomycin resistance marker, and later transformants were scored.

^c nd; Transformants were not detected.

Abbreviation: TF, transformants.

the production of the activator by competence-induced cells.

Repair of mutation by transformation

Table II demonstrates that the defect in the Tra-12 strain was recovered on the transformation using wild type DNA. After competence had been induced with the activator, Tra-12 cells were incubated with wild type DNA (Ery^r) in competence medium at 30°C for 1 hour (the first transformation). Then, fresh growth medium was added to the culture, and the cells were grown at 37°C for several hours to eliminate the residual competence and the activator activity in the culture fluid. To estimate the recovery of the defect on the first transformation, the culture was inoculated into fresh competence medium containing an excess amount of streptomycin resistant donor DNA (1 µg) (the second transformation), and then Str^r colonies were scored on agar plates (Table II). In the second transformation, Str^r colonies of Tra-12 were obtained only when the incompetent cells were transformed with wild type pneumococcal DNA in the first transformation, as indicated by the presence of Ery^r transformants. The number of Str^r transformants was affected by the amount of donor DNA in the second trans-

formation. Thus, the defect of Tra-12 was recovered on the transformation with wild type DNA.

DISCUSSION

In this paper we described the isolation and characterization of a pneumococcal mutant that is deficient in the production of activator (competence inducing factor) activity. The mutant showed the following characteristics: 1) The mutant Tra-12 did not spontaneously develop competence during culture growth (Fig. 1), but competence in these cells could be induced by the addition of the activator extracted from the wild type strain (Figs. 2, 3 and Table I). 2) No activator activity was extracted from Tra-12 cells during any time of culture growth (Fig. 1). 3) The lack of accumulation of the activator activity was observed in the competent Tra-12 cells induced by the addition of exogenous activator (Fig. 4). These facts indicate that the mutant Tra-12 is defective in the production of the active activator. The process of competence development assumed to proceed normally after the treatment of the mutant cells with exogenous activator. This assumption is supported by the finding of normal DNA bind-

ing activity of Tra-12 cells after incubation with the activator (Table I). Thus, Tra-12 is a novel mutant that can be added to the broad class of transformation deficient mutants.

In addition, it was observed that the competence induced by the activator decreased more rapidly in the Tra-12 culture than in the R6 culture (Fig. 4), and that Tra-12 cells needed more activator activity to obtain a saturating level of competence (Fig. 3). These findings suggest that the presence of the activator is necessary for immediate development and maintenance of competence, and support the speculation that the development of competence occurs in an autocatalytic manner.²⁾ The rapid decrease in competence in Tra-12 cells may affect the efficiency of the appearance of transformants. In fact, as can be seen in Table I, the number of transformants appeared in Tra-12 cells induced by the addition of the exogenous activator was smaller than that of R6, although Tra-12 cells bound almost the same amount of DNA as R6 cells. These may be caused by the decrease in the efficiency of the recombination process. With respect to this, it has been reported that resident DNA of competent cells of *Haemophilus influenzae* contain a larger amount of single-stranded regions than that of incompetent cells.¹⁹⁾ Thus, the decrease of competence in Tra-12 cells would reduce the single-stranded regions of resident DNA that are favorable for the interaction between resident DNA and transforming DNA.

It is very significant that the defect of the Tra-12 strain was recovered on transformation with wild type DNA. By using double transformation to isolate the fragment that can transform Tra-12 cells to the wild type, it

will be possible to clone the gene involved in the process of activator production.

Further analysis of this kind of mutant will allow elucidation of the process of induction of competence in genetic transformation of *S. pneumoniae*.

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