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Fate of Homospecific Transforming DNA Bound to *Streptococcus sanguis*

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The fate of [^{3}H]DNA from *Streptococcus sanguis str-r43 fus-s* donors in [^{14}C]*S. sanguis str-s fus-r1* recipients was studied by examining the lysates prepared from such recipients at various times after 1 min of exposure to DNA. The lysates were analyzed in CsCl and 10 to 30% sucrose gradients; fractions from the gradients were tested for biological activity and sensitivity to nucleases, subjected to various treatments and retested for nuclease sensitivity, and run on 5 to 20% neutral and alkaline sucrose gradients. The results demonstrate that donor DNA bound to *S. sanguis* cells in a form resistant to exogenous deoxyribonuclease is initially single stranded and complexed to recipient material. Donor DNA can be removed from the complex upon treatment of the complex with Pronase, phenol, or isoamyl alcohol-chloroform. Within the complex, donor DNA is relatively insensitive to S1 endonuclease but can regain its sensitivity by treatment with phenol. With time the complex moves as a whole to associate physically with the recipient chromosome. After a noncovalent stage of synapsis, donor material is covalently bonded to and acquires the nuclease sensitivity of recipient DNA, while donor markers regain transforming activity and become linked to resident markers.

Previous studies of the fate of radioactively or density labeled transforming DNA in recipients of *Streptococcus pneumoniae* (3, 4) and *Bacillus subtilis* (2) have essentially agreed upon the following points: (i) donor DNA is rendered single stranded upon uptake; and (ii) this surviving strand is covalently inserted into the recipient chromosome at a subsequent stage. Little is known, however, about the conditions that protect the surviving strand from degradation before integration and guide it to the recipient chromosome in a configuration permitting synapsis with a homologous sequence. In studies of the Challis strain of *S. sanguis*, which is closely enough related to undergo reciprocal transformations with *S. pneumoniae*, we have obtained evidence of the formation of a stable complex between single-stranded donor DNA and a recipient protein, which subsequently becomes associated with the resident chromosome. Within the complex the donor DNA is relatively insensitive to pancreatic deoxyribonuclease (DNase) I as well as the single-strand specific S1 endonuclease of *Aspergillus oryzae*. These studies were aided by a very high level of competence induced in *S. sanguis* cells, which permitted a 1 to 5% uptake of transforming DNA in 1 min, as well as by an efficient means of lysing *S. sanguis* recipients involving only a brief exposure to phage C lysin.

A preliminary report of these findings has been published (11).

MATERIALS AND METHODS

Bacterial strains. A streptomycin-sensitive, fusidic acid-resistant (*str-s fus-r*) and a streptomycin-resistant, fusidic acid-sensitive (*str-r fus-s*) mutant of the Challis strain of *S. sanguis* were used as recipient and donor, respectively.

Lysin. C phage-induced lysin was prepared by the method of Barkulis et al. (1) with the following modifications. The fraction of C phage-induced cell lysate of *Streptococcus* strain 26RP66 precipitated at 50% saturation of $(NH_4)_2SO_4$ and containing the bulk of lysin activity was centrifuged at 35,000 rpm for 3 h at 0 to 4°C to remove the phages. This crude lysin preparation was then desalted by filtration through a Sephadex G50 column, and the lysin activity was recovered in the void volume. This was followed by precipitation with $(NH_4)_2SO_4$ (20% saturation) and, after standing for 20 min at 0 to 4°C, the precipitate was collected by centrifugation, dissolved in buffer, centrifuged at 35,000 rpm for 2 h and frozen at −75°C.

Spheroplast formation and lysis. When cells were to be rendered spheroplasts, they were collected by centrifugation, washed twice with lysis buffer (0.1 M sodium phosphate [pH 6.1]-5 mM each of ethylenediaminetetraacetic acid [EDTA] and mercaptoethanol), and suspended in the same buffer containing 20% (wt/vol) sucrose. A 5% (vol/vol) lysin preparation was then added to the suspension, which was incubated at 37°C. The formation of intact spheroplasts was checked by quantitative conversion of gram-positive,

osmotically stable into gram-negative, osmotically sensitive units. Spheroplasts were lysed under a variety of conditions, to be described below, depending upon the purpose of the lysate. The rate of lysis depended primarily upon spheroplast density. Conditions under which lysis would occur included incubation at 0 to 4°C.

Transforming DNA. Frozen cultures of the *str-r43 fus-s* strain of *S. sanguis* were thawed, and the cells were pelleted by centrifugation and then suspended in 30 ml of M1 medium (0.4% yeast extract-1% neopeptone-0.85% NaCl) containing 0.75% glucose (wt/vol) and 10 to 15 μCi [*methyl*-^{3}H]thymidine per ml and incubated at 37°C. When the cell density reached 1×10^9 to 2×10^9 colony-forming units per ml (as estimated by optical density measurements in a Spectronic 20 colorimeter), the cells were centrifuged, washed twice in lysis buffer and suspended in 8 to 10 ml of the same buffer. Spheroplasts were prepared as described above, collected by centrifugation, and then lysed by mixing in 5 ml 1× SSC (0.15 M NaCl + 0.015 M Na-citrate) containing 1 mg of sodium dodecyl sulfate and 200 μg of proteinase K per ml, and incubated at 60°C for 1 h. The lysate was cooled and precipitated with 2 volumes of cold (4°C) ethanol. The precipitate was dissolved in 1× SSC. RNA was then digested with 100 μg of ribonuclease (RNase) per ml for 30 min at 37°C followed by digestion with proteinase K at 500 μg/ml in the presence of sodium dodecyl sulfate (1 mg/ml) for 3 to 4 h at 60°C. The lysate was then deproteinized by shaking with isoamyl alcohol-chloroform, and the DNA was finally precipitated with ethanol. The optical density ratio at 260 to 280 nm of a typical preparation ranged between 1.8 and 2.2, and the specific activity ranged between 1×10^5 and 2×10^5 cpm/μg of DNA.

Competence and transformation. Log-phase *str-s fus-r1* cultures, previously frozen in 10% glycerol at −75°C, were thawed at 37°C, diluted 16.6-fold in prewarmed competence medium (Pakula medium + 10% swine serum [12]) containing 0.1 μCi of [^{14}C]thymidine per ml, and incubated at 37°C. When the cultures had reached their maximal competence (usually 5×10^7 to 10×10^7 cells/ml), ^{3}H-labeled donor DNA was added for a final concentration of 2 to 5 μg/ml, and uptake was allowed to proceed for 1 min at 37°C. Mg^{2+}-activated DNase I (20 μg/ml of culture) was then added to terminate uptake, and incubation continued for 1 min. An equal volume of ice-cold M1 medium was then added, and the mixture was cooled in an acetone-ice bath. The cells were collected by centrifugation at 0 to 4°C, washed twice with cold M1 medium, suspended in the same medium, and then divided into fractions, which were either held on ice or incubated at 37°C for the desired length of time before lysis. After washing twice with lysis buffer, the cell pellet was suspended in 0.25 ml of lysis buffer containing 20% (wt/vol) sucrose. The lysis buffer removes acid-soluble donor DNA produced upon uptake. Purified C phage lysin was then allowed to act upon the cell suspension for 1 to 2 min at 37°C. When the lysates of transformed recipients were to be assayed for donor and recipient marker activity, 0.2 (or 0.4) ml was mixed with about 3×10^6 or 6×10^6 competent *fus-s str-s S. sanguis* cells in 2 (or 4) ml of NS medium (12) containing beef serum albumin (1% wt/vol), incubated for 15 to 20 min at 37°C, and then treated with DNase I. Platings were then made in nonselective media. Plates were overlayered after 2 h of incubation at 37°C with 50 μg of fusidic acid per ml to select for the *fus-r* marker, 100 μg of streptomycin per ml for the *str-r* marker, and a combination of the two antibiotics for the linked *str-r fus-r* markers. The *str* and *fus* loci exhibit 20% cotransfer, so that when *str-s fus-r* recipients are transformed with *str-r fus-s* DNA, genetic linkage of the recipient *fus-r* marker with the donor *str-r* marker in the lysate is indicated by a value of 0.16 in the ratio of *str-r fus-r* to *str-r* activity (probability of separation of *str-s* from *fus-r* in the first round of transformation multiplied by the probability of cotransfer of *str-r* with *fus-r* in the second round, or 0.8×0.2).

High-salt 10 to 30% sucrose gradient analysis. A 0.2-ml portion of a spheroplast suspension, prepared as described above, was layered onto 0.2 ml of a detergent-containing buffer [2 M NaCl, 2 mM EDTA; 2 mM mercaptoethanol, 0.02 M tris(hydroxymethyl)aminomethane (Tris) (pH 8.1), 1% Brij 58, 0.6% sodium deoxycholate], itself on top of 4.6 ml of a 10 to 30% neutral sucrose gradient made in buffer containing 1 M NaCl, 0.01 M Tris, 1 mM EDTA, and 1 mM mercaptoethanol (pH 8.1) resting on 0.2 ml of a 60% sucrose (wt/vol) cushion. Lysis took 10 to 20 min at 0 to 4°C. The gradients were sedimented at 17,000 rpm for 40 min at 4°C in an SW 50.1 rotor. Fractions were collected from the bottom of the tube. Alternate fractions were examined for acid-precipitable radioactivity, and appropriate unprecipitated fractions were pooled to contain the fast- and slowly sedimenting DNA. The pooled fractions were dialyzed extensively against cold 0.15 M NaCl for 16 to 18 h at 0 to 2°C and then used for biological assay of the donor or resident marker, nuclease sensitivity, and sedimentation studies.

CsCl gradient analysis. Spheroplasts prepared as described above were diluted two- to threefold with buffer (40 mM potassium phosphate buffer, pH 11). A 0.1-ml portion of 6% Sarkosyl made up in the same buffer was then added, and the mixture was gently shaken by vortexing for 30 s, at which time lysis was complete. Saturated CsCl was then added to the lysate to a final density of 1.6 g/cm^3. A 1-ml fraction of this mixture was gently layered on top of a step CsCl gradient, the bottom and middle layers having densities, respectively, of 1.8 and 1.7 g/cm^3. This step gradient was then centrifuged at 30,000 rpm for 16 to 18 h at 18°C in an SW 50.1 rotor to form a linear gradient. Fractions were collected from the bottom either directly on glass filter disks (GF/A) or in tubes. Filters were washed with cold 5% trichloroacetic acid followed by cold alcohol, dried and then counted. Appropriate fractions collected in tubes were pooled, dialyzed against cold 0.15 M NaCl, and used for the assay of biological activity and nuclease sensitivity.

Five to twenty percent neutral and alkaline sucrose gradient analysis. Fractions from 10 to 30% sucrose gradients containing the slowly sedimenting ^{3}H-labeled donor DNA and fast-sedimenting ^{14}C-labeled recipient DNA were pooled, dialyzed against two changes of cold 0.15 M NaCl and concentrated,

and 0.2 ml from each was layered on top of either a 5-ml neutral 5 to 20% sucrose gradient (1 M NaCl-1% Sarkosyl-0.01 M EDTA [pH 8.1]) or a 5-ml 5 to 20% alkaline sucrose gradient (0.7 M NaCl-0.3 M NaOH-0.01 M EDTA-1% Sarkosyl [pH 12.3]). The gradients were sedimented at 30,000 rpm for 2 h at 18°C in an SW 50.1 rotor. Fractions were collected, precipitated, and counted as above. ^{14}C-labeled T4 DNA (double-strand mass of 110 megadaltons (Mdal); single-strand mass of 55 Mdal) was used as the reference DNA. The molecular weight of ^{3}H-labeled donor native and denatured DNA and that of complexed donor DNA before and after deproteinization were calculated by the method of Studier (14).

BNDC chromatography. Spheroplasts prepared as described above were diluted with buffer (1 mM Tris-1 mM EDTA-0.3 M NaCl [pH 8.1]) to a final volume of 4 to 5 ml. Sarkosyl was then added to a final concentration of 1 mg/ml and, after thorough mixing, the lysate was loaded on to a benzolyated, naphthoylated diethylaminoethane-cellulose (BNDC) column (1-ml bed volume in a 2-ml plastic syringe). DNA was eluted from the column by applying a linear gradient of 0.3 to 1 M NaCl and 0 to 2% caffeine prepared in 1 mM Tris-1 mM EDTA (pH 8.1). A total of 50 1-ml fractions were collected at a flow rate of 0.5 ml/min, and each fraction was precipitated with 10% trichloroacetic acid. After standing at 0 to 4°C for 16 to 18 h, the precipitate was collected on filters (GF/A), washed with 5% trichloroacetic acid and alcohol, and then dried and counted.

Sephadex G50 column chromatography. A 2- to 5-ml portion of the sample to be analyzed was loaded onto a Sephadex G50 column (bed volume, 100 ml; void volume, 40 ml; elution buffer, 1× SSC). Fractions of 3 ml were collected at a flow rate of 1 ml/min. From each fraction, 0.2 ml was spotted on GF/A filters in duplicate. One set of filters was washed with cold 5% trichloroacetic acid and cold alcohol. All the filters were dried, and the radioactivity was counted.

Nuclease assays. All nuclease assay mixtures were assembled on ice. Pancreatic DNase I (Worthington Biochemicals Corp.) assays contained in 1 ml; 0.15 M NaCl, 1 mM $MgCl_2$, and 10 μg of enzyme. *A. oryzae* S1 endonuclease (Miles Laboratories, Inc.) assays contained in 1 ml; 33 mM acetate buffer (pH 4.5), 3.5 μM $ZnCl_2$, and 2 μg of enzyme. Micrococcal nuclease (Worthington) assays contained in 1 ml; 10 mM sodium borate buffer (pH 8.8), 0.5 mM $CaCl_2$, and 10 μg of enzyme. An amount of the fraction to be tested was added to contain 400 to 1,200 cpm. Assays were incubated at 37°C for 15 min. The reaction was terminated by adding 1 ml of cold 10% trichloroacetic acid. Trichloroacetic acid-insoluble precipitates were collected on GF/A filters, and radioactivity was counted.

RESULTS

Changes in sedimentation and biological properties of bound donor DNA. [^{14}C]thymidine-labeled competent recipient cells bound about 1 to 5% of the input donor [^{3}H]DNA in 1 min at 37°C in a form insensitive to DNase I. When analyzed in high-salt, 10 to 30% sucrose density gradients, lysates of these cells made immediately after uptake showed that 15 to 20% of the donor counts cosedimented with the recipient DNA, which because of its association with RNA and protein (8, 15), sedimented at or near the bottom of the gradient (Fig. 1). The remaining donor counts were located in a broad band within the upper half of the gradient. Either native or denatured DNA, subjected to similar analysis after being mixed with the spheroplasts of cells not exposed to donor DNA but processed under similar conditions, was recovered only at the very top of the gradient. Lysates of cells that were allowed to incubate at 37°C for various lengths of time after uptake revealed that, with increasing time of incubation, an increasing fraction of the donor DNA cosedimented with the recipient DNA until, at 20 min of incubation, all of the donor counts cosedimented with the recipient (Fig. 1). Recovery of donor counts bound to DNase-treated, lysis buffer-washed cells was over 95% from all of the gradients, regardless of the length of incubation of the cells. Moreover, during the entire period

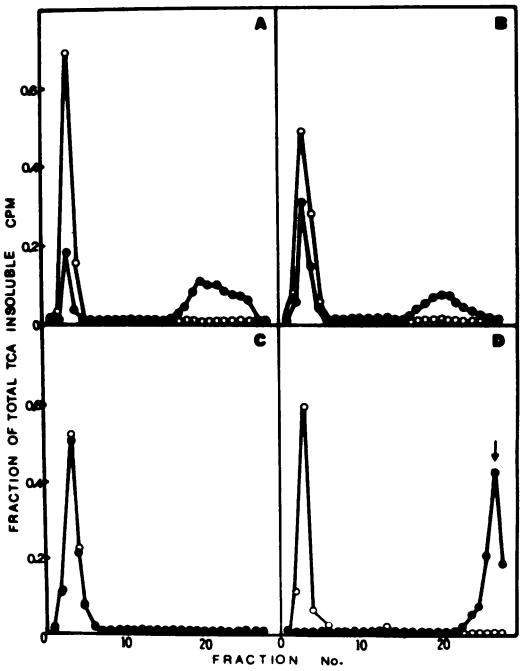

FIG. 1. *Preparative high-salt, 10 to 30% neutral sucrose density gradient analysis of donor and recipient DNA. Spheroplasts were prepared and analyzed as described in the text. Sedimentation profiles of ^{3}H-labeled donor (●) and ^{14}C-labeled recipient (○) DNA 1 min (A), 5 min (B), and 20 min (C) after termination of uptake of donor DNA by competent recipient cells. (D) Sedimentation profile of ^{3}H-labeled native DNA after mixing with spheroplasts made from ^{14}C-labeled, untransformed recipient cells. Arrow shows the position of denatured ^{3}H-labeled donor DNA in a similar gradient.*

of incubation, none of the donor counts was found in acid-soluble form.

Concomitant with the binding of donor DNA in DNase-resistant form is loss of its biological activity, indicating its altered physical state upon uptake. This conclusion is derived from the results recorded in Table 1. No donor marker activity was detected in slowly sedimenting fractions of the sucrose gradient, except for an amount that was accounted for by the slight contamination of these fractions with recipient DNA. Donor marker activity was found in the fast-sedimenting fractions where it increased with time of incubation after uptake. In the fast-sedimenting fractions, linkage was also observed between the donor and recipient markers, indicating that the donor marker had been genetically integrated with recipient DNA.

Bound donor DNA is single stranded but complexed to recipient cell components. Analysis of the supernatant surrounding the cells 15 min after they had been exposed continuously to donor DNA revealed that an amount of DNA equivalent to the amount recovered from the cells in a DNase I-resistant state was present in the medium in acid-soluble form (Fig. 2). That this breakdown was a specific consequence of uptake and not due to any nonspecific breakdown of donor DNA in the presence of cells was shown by the absence of acid-soluble donor counts upon incubation of the DNA with noncompetent cells for the same length of time. Since in this experiment contact between transforming DNA and recipients was not limited to 1 min, as in our other experiments, we devised a different procedure to determine the extent of solubilization of donor DNA bound after 1 min of uptake. We found that washing with SSC does not, but that washing with lysis buffer does, remove acid-soluble donor material bound to *S. sanguis* cells immediately after uptake. All of the acid-soluble material not removable with SSC appeared in the culture medium by 15 to 30 min of incubation at 37°C. By utilizing the procedure of washing with lysis buffer, we were able to show that solubilization of bound donor DNA almost immediately follows uptake and that approximately half of the bound DNA is solubilized (Table 2).

This result suggests an uptake process in which one strand of donor DNA is degraded as the other complementary strand is bound (4). This interpretation was supported by BNDC chromatography of lysates of ^{14}C-labeled cells exposed to ^{3}H-labeled donor DNA. The elution profiles of recipient and donor DNA are presented in Fig. 3. Soon after uptake almost all of the ^{3}H-labeled donor DNA bound in the DNase I-resistant form eluted from the column with salt and caffeine concentrations similar to those that eluted single-stranded donor DNA chromatographed after being mixed with lysates of competent ^{14}C-labeled cells that were never exposed to transforming DNA. This elution profile was entirely unlike that of native donor DNA mixed with lysates of untransformed recipients;

TABLE 1. *Biological activity of fast- and slow-sedimenting fractions from lysates sedimented in neutral sucrose gradients*

Time incubated (min)	Fractions	^{3}H cpm/assay	No. of transformants/ml		
			str-r[a] (donor)	*fus-r str-r*[b]	*fus-r str-r/str-r*[c]
1	Fast[d]	1,010	200	25	0.13
	Slow	3,260	53	3	
5	Fast[d]	2,930	1,450	230	0.16
	Slow	1,190	116	16	
20	Fast[d]	4,590	3,670	660	0.18
	Slow	150	50	12	
Control[e]	Fast[d]	0	11	0	
	Slow	3,130	2,430	3	

[a] Two 0.5-ml volumes were plated, with the exception that a 0.1- and a 0.5-ml volume were plated for the fast-sedimenting fraction from the 20-min sample and the slow-sedimenting fraction from the control sample. Variation between the two samples was always less than 10%.

[b] Volumes of 0.5 and 1.0 ml were plated. Average of the two samples is presented.

[c] Observed ratio of *fus-r str-r* transformants is to be compared with the ratio expected if the *str-r* marker were genetically integrated into the recipient genome. This expected value is 0.16, calculated as the product of the previously determined cotransfer of the *fus* and *str* loci (0.2) and the probability of separation of the *fus-s* and *str-r* markers from the donor DNA upon integration (0.8).

[d] Biological activities in fast-sedimenting fractions were nonsaturating in the test system employed. ^{14}C and *fus-r* transforming activity was always ≥90% in the fast fraction (11).

[e] Control is ^{3}H-labeled transforming donor DNA mixed with a lysate of ^{14}C-labeled recipient cells not previously exposed to DNA.

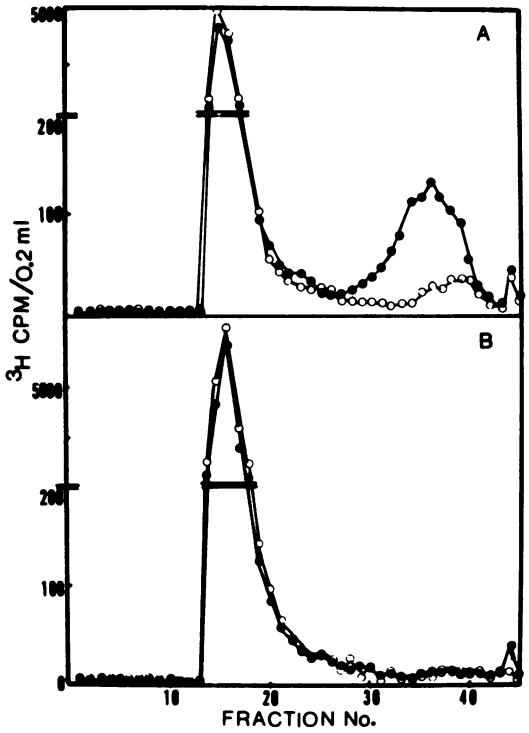

FIG. 2. *Sephadex G50 column chromatography. Competent or non-competent recipient cells were exposed to ^{3}H-labeled str-r43 DNA for 15 min at 37°C. Uptake was terminated by transferring the tubes to an acetone-ice bath. Cells were pelleted at 0 to 4°C, and 2 ml of the supernatant was then loaded onto a Sephadex G50 column. Elution and analysis were as described in the text. The pellet was suspended in 1 ml of 0.15 M NaCl containing 20 μg of DNase I and 10^{-3} M $MgCl_2$. The mixture was incubated at 37°C for 5 min. The cells were then collected on a GF/A filter, washed with 1× SSC, and counted. A total of 5% (16,000 cpm) of the donor input label was bound in DNase I-resistant form by competent recipient cells, whereas the noncompetent cells bound less than 0.02% of the input label. (A) Competent cell supernatant; (B) noncompetent cell supernatant. Total ^{3}H counts per minute (●); trichloroacetic acid-precipitable ^{3}H counts per minute (○). Total number of trichloroacetic acid-soluble counts in fractions 25 to 45 in (A) = 15,000 cpm.*

native donor DNA eluted with recipient DNA at relatively low NaCl-caffeine concentrations (Fig. 3). After 20 min of incubation at 37°C following uptake, the elution profile of donor DNA was identical to that of the recipient [^{14}C]DNA, indicating that the donor DNA is presumably covalently linked to the recipient DNA at this time.

Nevertheless, the bound donor DNA in single-stranded form was not free but tightly complexed with a cellular component, as shown by profiles of donor and recipient DNA in CsCl density gradients. Immediately after uptake, the peak of donor [^{3}H]DNA coincided with the peak of ^{14}C-labeled recipient DNA, but whereas the recipient DNA was contained in a sharp band, the band of donor DNA was broader and had a pronounced shoulder on the lighter side of the gradient (Fig. 4). Longer runs to 65 h in the gradient (not shown) did not alter this shoulder. The shoulder progressively disappeared, however, with time of incubation after uptake until, at 20 min, the donor and recipient DNA profiles were superimposable. Denatured ^{3}H-labeled donor DNA, when mixed with a ^{14}C-labeled cell lysate and subjected to similar analysis, banded as expected at a density position greater than that of recipient DNA. The bulk of donor DNA in cell lysates 1 min after uptake, although unassociated with recipient DNA, nevertheless sedimented more rapidly in a preparative 10 to 30% sucrose gradient than did free native or denatured transforming DNA (Fig. 1). Fractions of such a gradient containing donor DNA unassociated with recipient DNA were pooled, dialyzed, divided into four parts, treated as indicated in Fig. 5, and sedimented in 5 to 20% neutral sucrose gradients. Donor [^{3}H]DNA sedimented as a 35S molecule, but treatment with phenol, isoamyl alcohol-chloroform, or Pronase changed this value to about 25S (ca. 1.4 Mdal for single-stranded DNA), indicating that, although insensitive to the high salt concentration and Sarkosyl in the gradient, the complex is disrupted upon treatment with any of these deproteinizing agents, releasing as a result DNA that sediments at a rate similar to that of single-stranded donor DNA molecules 1.5 Mdal in size (Fig. 5). Similar treatment, however, did not

TABLE 2. *Solubilization of donor DNA bound to S. sanguis recipients*[a]

Incubation at 37°C after uptake termination (min)	Total cpm bound in DNase-resistant form after uptake termination	Trichloroacetic acid-insoluble cpm in lysate	% Solubilization of initially bound cpm
0	32,880	17,410	47
30	32,880	16,240	51

[a] *S. sanguis* recipients were exposed to [^{3}H]DNA for 1 min at 37°C, then to DNase I for 1 min. A portion of the cells was collected on a GF/A filter, washed with 1× SSC, and measured for total counts per minute bound in DNase-resistant form. The remainder was divided into two equal parts: (i) those held on ice for 30 min; and (ii) those incubated at 37°C for 30 min. The cells were washed with lysis buffer for removal of all bound trichloroacetic acid-soluble counts and lysed, and the lysate was measured for trichloroacetic acid-insoluble radioactivity.

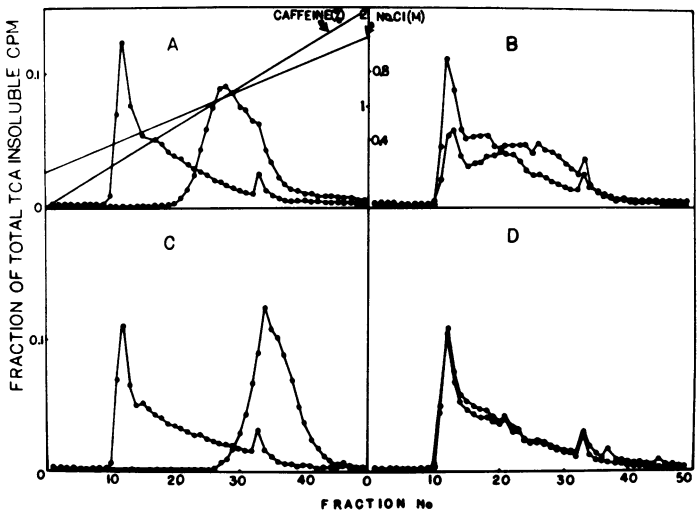

FIG. 3. *BNDC chromatography. Lysates were prepared as described in the text. Elution profile of ^{3}H-labeled donor (●) and ^{14}C-labeled (○) recipient DNA 1 min (C) and 20 min (D) after termination of donor DNA uptake by competent recipient cells. Elution profile of ^{3}H-labeled denatured (A) and native (B) DNA mixed with lysates of ^{14}C-labeled competent, untransformed recipient cells. Recovery of ^{3}H and ^{14}C label from the columns was between 80 and 85%.*

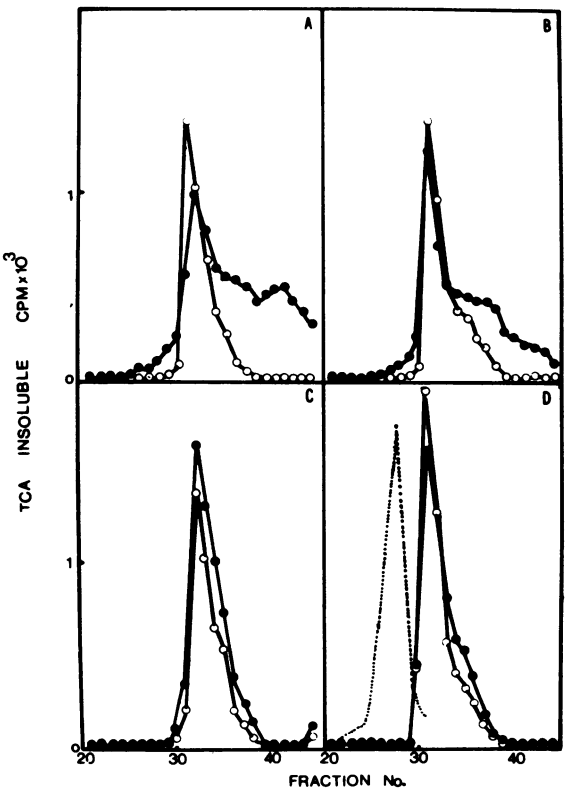

FIG. 4. *CsCl equilibrium density gradient analysis. Lysates were prepared and analyzed as described in the text. Density profiles of ^{3}H-labeled donor (●) and ^{14}C-labeled recipient (○) DNA 1 min (A), 5 min (B), and 20 min (C) after termination of uptake of donor DNA by competent recipient cells. Density profile of lysates (D) made up of native (—) or denatured (· · · · ·) ^{3}H-labeled donor DNA mixed with spheroplasts of ^{14}C-labeled competent, untransformed recipient cells prior to lysis and analyzed as described in Materials and Methods. Recovery of ^{3}H and ^{13}C label from the gradients was over 95%.*

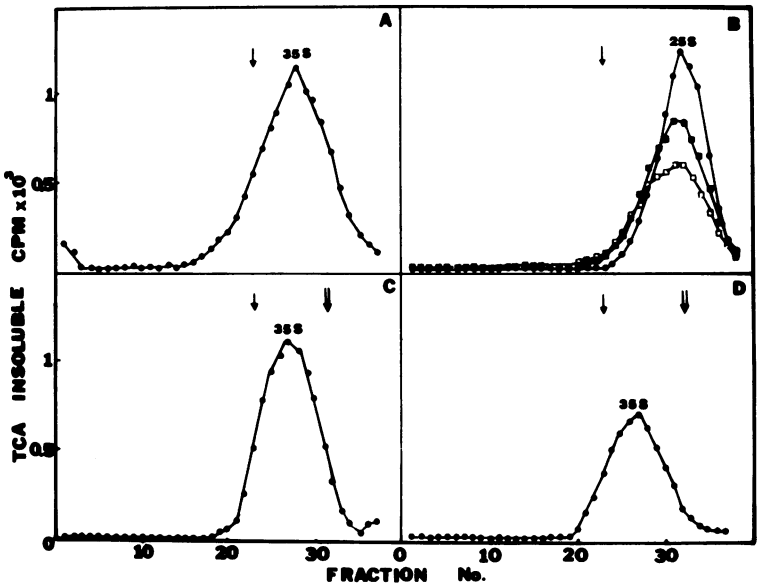

FIG. 5. *Zone sedimentation in 5 to 20% neutral sucrose density gradients of slowly sedimenting ^{3}H-labeled donor DNA removed from 10 to 30% neutral sucrose gradients (Fig. 1). Sedimentation profile of complexed donor DNA before (A) and after (B) treatment with phenol saturated with 0.01 M Tris buffer (pH 10), 1 M NaCl, and 1% sodium dodecyl sulfate (●), 100 μg of Pronase (predigested) (■) per ml or chloroform-isoamyl alcohol (□). Native ^{3}H-labeled DNA mixed with spheroplasts of untransformed ^{14}C-labeled recipient cells was sedimented in 10 to 30% neutral sucrose gradients as described in the text. Fractions containing the slowly sedimenting native ^{3}H-labeled DNA (Fig. 1D) were pooled, dialyzed, and concentrated, and 0.2 ml was layered on top of a 5 to 20% neutral sucrose density gradient before (C) and after (D) treatment with phenol, sedimented, and analyzed as described in the text. Single arrow indicates the position of reference ^{14}C-labeled native T4 DNA in the gradient. Double arrow (C and D) indicates the position of denatured ^{3}H-labeled DNA, previously sheared to 1.5 Mdal in size, before and after treatment with phenol, respectively.*

alter the sedimentation of either native or denatured DNA in the gradients. In alkaline gradients the DNA-recipient complex was unstable, and the dissociated DNA sedimented with an S value (16S) that corresponded to a single-strand length of about 1.7 Mdal as compared with that of about 8 to 10 Mdal for native donor DNA (Fig. 6).

DNA in the complex was relatively insensitive to DNase I and single strand-specific *A. oryzae* S1 endonuclease, but completely sensitive to micrococcal nuclease (Table 3). Deproteinization of the complex significantly increased its susceptibility to degradation by S1 endonuclease. Susceptibility to DNase I also increased, although to a lesser extent (Table 3).

Association of the donor DNA-recipient protein complex with the chromosome. The portion of the bound donor label that is already associated with recipient DNA 1 min after uptake was further analyzed by resedimentation in 5 to 20% neutral sucrose gradients (Fig. 7). The association of donor and recipient DNA resisted the high salt concentration and Sarkosyl present in the gradient. However, deproteinization dissociated the association causing the donor DNA to sediment separately from recipient DNA and at a rate corresponding to that of deproteinized donor DNA unassociated with the chromosome. Treatment with phenol did not change its sedimentability in alkaline sucrose gradients. Donor DNA associated with the recipient DNA 1 min after uptake exhibited the same sensitivity to DNase I and S1 endonuclease as did unassociated donor DNA (Table 3). Moreover, after phenol treatment, susceptibility to these nucleases changed similarly for donor DNA that was associated and unassociated with the chromosome (Table 3). Donor DNA associated with the recipient chromosome 1 min after uptake is, therefore, not yet covalently linked to recipient DNA but is still in a nuclease-resistant, phenol-sensitive complex. By 20 min after uptake, however, when all of the donor DNA had cosedimented with recipient DNA in preparative 10 to 30% sucrose gradients, the association was of a different character. When the fast-sedimenting material was pooled and subjected to analysis by sedimentation in neutral or alkaline 5 to 20% sucrose gradients, the donor DNA cosedimented

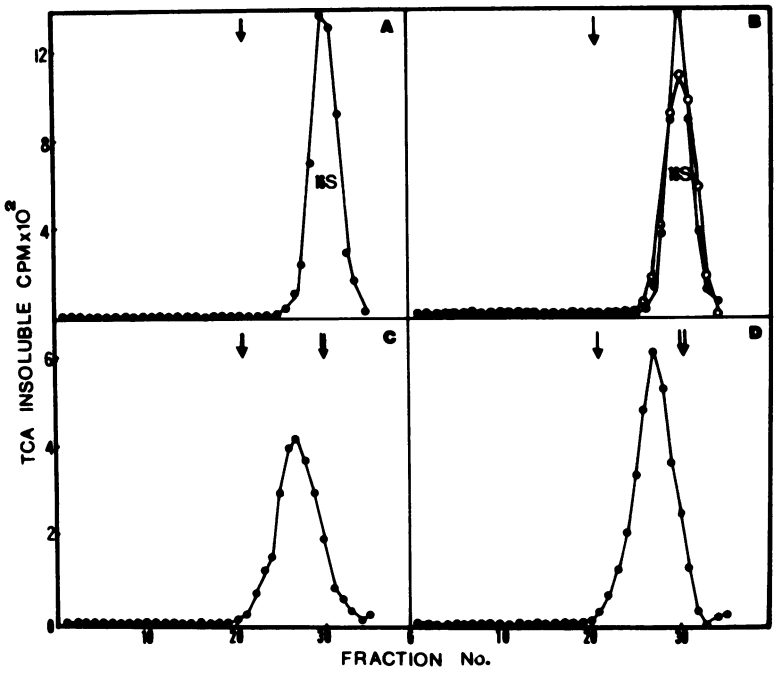

FIG. 6. *Zone sedimentation in 5 to 20% alkaline sucrose density gradients. Untreated and treated donor DNA fractions were the same as those described in the legend to Fig. 5. A 0.2-ml portion of each was layered on top of a 5 to 20% alkaline sucrose gradient, sedimented, and analyzed as described in the text. Sedimentation profile of complexed donor DNA before (A) and after (B) treatment with phenol (●) or Pronase (○). Sedimentation profiles of native ^{3}H-labeled donor DNA recovered from 10 to 30% neutral sucrose gradients as described in the legend to Fig. 5 before (C) or after (D) treatment with phenol. Single arrow shows the position of reference ^{14}C-labeled T4 native DNA in the gradients. Double arrow indicates the position of denatured ^{3}H-labeled donor DNA of 1.5-Mdal size in similar gradients.*

with recipient DNA at about 70S, and neither phenol nor alkaline conditions could dissociate it (Fig. 8). This integrated, biologically active donor DNA showed little sensitivity to S1 endonuclease before or after treatment with phenol and showed the same sensitivity to DNase I as did the recipient DNA (Table 3).

DISCUSSION

Acid-soluble donor DNA counts equivalent in amount to those cell-bound in the acid-insoluble form are produced by *S. sanguis* recipients after exposure to transforming DNA and eventually appear in the medium (Fig. 2). The equivalence between the amount of acid-soluble material released and the amount of acid-insoluble material bound in the DNase-resistant form suggests that only one of the two donor DNA strands initially bound is conserved on uptake; this is similar to the situation in *S. pneumoniae* where free single-stranded DNA is detectable in lysates of recipients soon after uptake (4), and an equivalent amount of acid-soluble donor DNA is found in the surrounding culture medium (5). That the bulk of the donor DNA in *S. sanguis* recipient lysates 1 min after uptake shows the binding affinities to BNDC characteristic of single-stranded DNA supports this view. However, in *S. sanguis* the donor DNA strand retained on uptake is tightly complexed with a cellular component or components, as has been reported for *B. subtilis* (9). This is indicated by the following facts.

(i) One minute after uptake, about 15 to 20% of the trichloroacetic acid-insoluble donor label in the recipient lysates cosediments with the recipient DNA which, due to its folded and supercoiled conformation (8, 15), sediments at a very fast rate in 10 to 30% neutral sucrose gradients. The remaining 80 to 85% of the donor label sediments at a much slower rate (which is nevertheless faster than that of either native or denatured donor DNA mixed in lysates of untransformed recipients).

(ii) The slowly sedimenting material has a buoyant density lower than that of denatured donor DNA.

(iii) Deproteinization of this material by Pronase, phenol, or chloroform-isoamyl alcohol treatment releases the DNA, as evidenced by

TABLE 3. *Relative sensitivity of donor DNA to nucleases after uptake by S. sanguis*

Sample no.[a]	Min after uptake	Phenol treatment	Fractions	% Sensitivity		
				DNase I	S1 Nuclease	Micrococcal nuclease
1	1	−	Slow	19	26	98
2	1	+	Slow	33	62	ND[b]
3	1	−	Fast: Donor	19	16	100
		−	Recipient	82	0	100
4	1	+	Fast: Donor	34	68	ND
		+	Recipient	79	0	ND
5	20	−	Fast: Donor	85	14	ND
		−	Recipient	84	13	ND
6	20	+	Fast: Donor	83	6	ND
		+	Recipient	85	7	ND
7		−	Slow denatured	43	54	99
8		+	Slow denatured	45	74	ND
9		−	Slow native	76	0	98
10		+	Slow native	67	8	ND
11		−	Denatured	46	85	100
12		−	Native	70	12	100

[a] Samples 1 through 6 were obtained from lysates of cells exposed to donor DNA for 1 min and incubated for either 1 or 20 min uptake, as indicated, and run on 10 to 30% sucrose gradients. Slow- and fast-sedimenting fractions were separately pooled. Samples 7 through 10 were obtained by mixing differentially labeled denatured and native DNA with lysates of cells not previously exposed to DNA, and then running on 10 to 30% sucrose gradients from which the slow-sedimenting fractions were pooled. Samples 1 through 10 were purified by banding in CsCl as described in the text. CsCl fractions containing radioactive label were pooled, dialyzed, and then used for enzyme assays. Samples 11 and 12 were purified denatured and native DNA controls.

[b] ND, Not done.

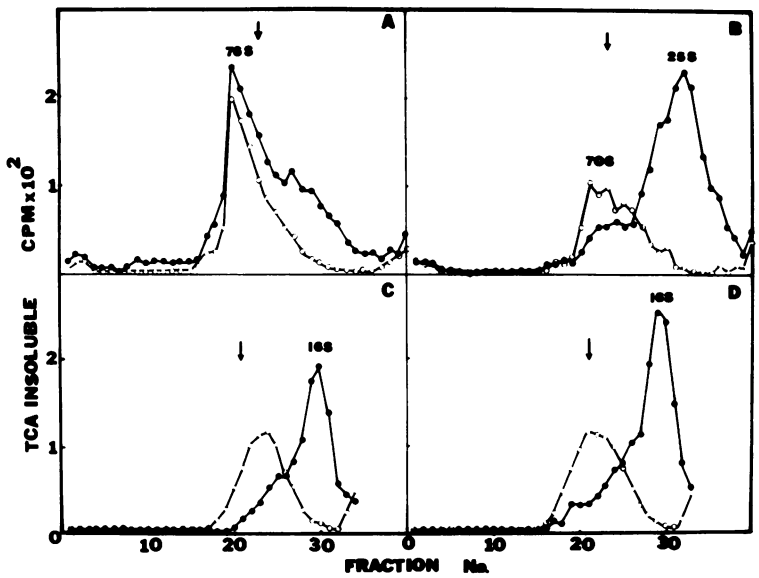

FIG. 7. *Zone sedimentation in 5 to 20% neutral or alkaline sucrose density gradients. Fractions from 10 to 30% neutral sucrose gradients containing the fast-sedimenting recipient and donor DNA complex formed 1 min after termination of donor DNA uptake were pooled and dialyzed, and 0.2 ml was layered on top of neutral (A and B) or alkaline (C and D) 5 to 20% sucrose density gradients before (A and C) or after (B and D) treatment with phenol, sedimented, and analyzed as described in the text.* ^{3}H*-labeled donor* (●) *and* ^{14}C*-labeled recipient* (○) *DNA. Arrows indicate the position of* ^{14}C*-labeled T4 DNA in similar gradients.*

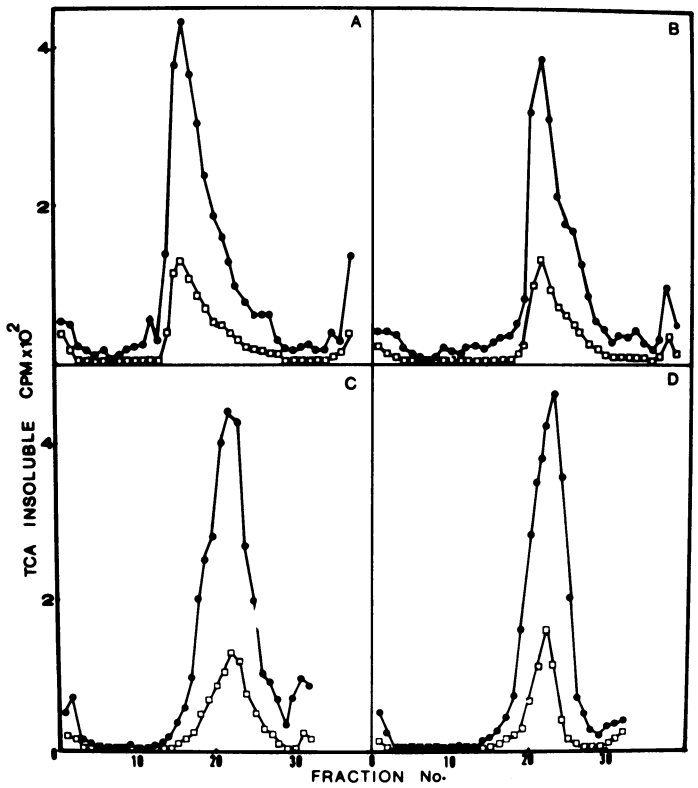

FIG. 8. *Zone sedimentation in 5 to 20% neutral or alkaline sucrose density gradients. Fractions from 10 to 30% neutral sucrose containing the fast-sedimenting recipient and donor DNA complex formed 20 min after termination of donor DNA uptake were pooled and dialyzed, and 0.2 ml was layered on top of neutral (A and B) or alkaline (C and D) 5 to 20% sucrose density gradients, before (A and C) or after (B and D) treatment with phenol, sedimented, and analyzed as described in the text.* ^{3}H*-labeled donor* (●) *and* ^{14}C*-labeled recipient* (□) *DNA.*

the reduced rate of sedimentation of the donor label and the similarity of this rate to the sedimentation rate of denatured input DNA sheared to about the same size (1.5 Mdal) as the donor DNA released by alkali. These findings indicate that the component(s) to which donor DNA is complexed is protein.

(iv) The slowly sedimenting donor material is in a biologically eclipsed state and is relatively insensitive to both DNase I and S1 endonuclease. The sensitivity to nucleases increases after deproteinization with phenol to almost the level of pure denatured donor DNA, similarly mixed in a competent (but untransformed) cell lysate.

That formation of the donor DNA-recipient protein complex represents an early step of transformation is indicated by the fact that the amount of this complex gradually declines with increasing length of incubation of transformed cells before lysis until all of the donor material originally bound in acid-insoluble form becomes associated with recipient DNA. The location of the complex within the recipient cell has been suggested by the results of centrifuging in a cold temperature, early after uptake, unlysed recipients treated with the wall-removing C-phage lysin in hypertonic medium (11). Most of the donor counts remain in the supernatant of the pelleted spheroplasts and not in the pellet where recipient counts are found, indicating that the bulk of the donor DNA shortly after uptake remains external to the cell membrane or readily removable from it. A similar observation was reported earlier in *B. subtilis* (6) but later disputed (16). Association of the complexed donor DNA with the resident chromosome appears to be a temperature-dependent process because cells placed on ice 1 min after uptake, but not lysed until 4 h later, were no different with respect to the proportion of donor label sedimenting with recipient DNA than those placed on ice for only 30 min (17 to 21% in the former case as opposed to 18 to 20% in the latter).

The temperature dependence of the change in

donor DNA from its early state to its final chromosomal location is also reflected in the gradual appearance of biological activity of the eclipsed donor marker in recombinant DNA over a 20-min period of incubation at 37°C after uptake. The formation of recombinant DNA (in which the donor marker is linked to the recipient marker) lags, however, behind the physical association of donor and recipient DNA (Table 1). An early step in this association does not involve a covalent bond, as is evidenced by the separability of the donor DNA from the chromosome by phenol or alkali treatment (Fig. 7) of the fast-sedimenting material in cell lysates immediately after uptake. Also, in this early stage of association, the donor DNA shows the same sensitivity to nucleases and the same size as when unassociated with recipient DNA. Therefore, we believe the donor DNA-recipient protein complex is transported in its entirety and is intact when physical association with the recipient chromosome occurs. The physical association, however, must be firm enough to resist the Sarkosyl and high salt concentration with which the lysates are treated in sucrose gradient analysis, and it is probably nonionic. Hence, we regard this early stage of physical association to represent the synapsed state of donor DNA base paired to recipient DNA but not covalently linked to it.

The final stage of association with the resident chromosome involves covalent linkage, at which time transforming activity of the donor marker is restored, genetically recombinant DNA is formed, and nuclease sensitivity and sedimentation properties of donor DNA become inseparable from those of recipient DNA.

We have, therefore, delineated at least three steps in the transformation of *S. sanguis*: (i) formation of a complex between single-stranded donor DNA and recipient protein(s). This step may be concomitant with uptake if the binding of DNA to specific receptor sites on the cell membrane, which causes degradation of one of the donor strands, were the process in which the complex is formed. If this were the case, one should find that the DNA-binding protein produced (or unmasked) in transformation-competent recipients is a part of the purified complex. (ii) Physical association between donor and the recipient DNA. This association may depend upon base sequence homology between donor and recipient DNA for both its initiation and maintenance. In the next paper (10) we shall present evidence that heterology in base sequence decreases the rate and stability of the association. (iii) Genetic integration of donor DNA into the recipient chromosome, which presumably requires the enzymatic machinery of recombination.

It is interesting that Shoemaker and Guild (13) have postulated that the recovery of genetic activity in eclipsed donor DNA in *S. pneumoniae* occurs in two discrete steps, each apparently possessing a characteristic activation energy. The association and integration steps, well resolved in the *S. sanguis* system, may very well constitute the two temperature-dependent processes postulated for *S. pneumoniae*.

The initial formation of the donor DNA-recipient protein complex is hardly likely to be a fortuitous outcome of the binding of donor DNA in the DNase-resistant form. The complex is probably significant for several reasons. First, the survival of donor DNA in the cell is probably enhanced as a consequence of complex formation. Second, association of the donor DNA with the chromosome probably depends upon its complexed nature. As we have seen, the complex appears to persist as the constituent DNA synapses with the recipient chromosome. Finally, it is possible that the protein component(s) of the complex help to stabilize synapsis, perhaps by maintaining the donor DNA in a configuration optimal for base pairing with the complementary recipient strand. The finding that phenol treatment of the associated donor and recipient DNA before integration causes dissociation supports this idea. A complex similar in some ways to the one we have described in this paper has recently been found in *S. pneumoniae* (7).

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