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Chromosomally Integrated Conjugative Plasmids Are Common in Antibiotic-Resistant *Haemophilus influenzae*

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Twenty-three highly antibiotic-resistant strains of *Haemophilus influenzae* and two of *Haemophilus parainfluenzae* without detectable large plasmids were examined for conjugative transfer of their resistance to *H. influenzae* strain Rd or to other strains. Very inefficient transfer was observed for 18 *H. influenzae* strains and 1 *H. parainfluenzae* strain. All *H. influenzae* transcipients carried a large plasmid, and they were in turn efficient donors of their resistances in standard conjugation crosses with isogenic recipients. This was not seen for the *H. parainfluenzae* transcipients. It is concluded that most of the original antibiotic-resistant cultures carried an integrated conjugative R plasmid which had been excised in a few cells in each population. It was these cells which transferred resistance in the primary crosses.

I have reported recently that of 29 isolates of *Haemophilus influenzae* highly resistant to ampicillin, chloramphenicol, or tetracycline 16 strains did not possess a detectable large plasmid (17). Since then, seven similar strains have been obtained. Based on direct physical evidence described for two strains (17), it is now believed for all 23 isolates that their resistances have a chromosomal location. In a recent unrelated experiment involving an Rd strain transformed to Tet^r with DNA from NT1261, it was by chance discovered that antibiotic resistance was (very infrequently) transferred to another Rd strain when the two strains were grown up in a mixed colony on an agar surface. Some of the transcipients carried a large plasmid and are now believed to be transconjugants. This new mode of transfer was studied for all 23 original isolates or their Rd transformants (Table 1). It will be shown in this report that at least 18 of them carry an integrated conjugative resistance transfer plasmid.

MATERIALS AND METHODS

Strains. In Table 1 are listed the antibiotic-resistant *Haemophilus* strains in which no large plasmid can be detected. More information about most of these strains can be found in a previous publication (17). Strains Sb1057, NT1760, and *H. parainfluenzae* 1662 and 1761 carry one or more small cryptic plasmids. Recipient strains used in the various crosses were *H. parainfluenzae* 700 (strain Boss, obtained from Grace Leidy), *H. influenzae* NT (Reid), NT (960) (see ref erence 17), and the BC200 derivative (2) of the widely used *H. influenzae* strain Rd (1). The *rec-1* mutant was obtained from Jane Setlow (16; her DB117). The mutation is very similar to *recA* mutations in *Escherichia coli* (see reference 12 for a detailed phenotypic description), and it is here called *recA1*. The *recA*$^+$ gene product is believed to control generalized recombination, and in *H. influenzae* it controls genetic transformation. Other genetic markers have been described (17, 18). Strains designated Sb carry a type b capsule, whereas NT isolates are unencapsulated (14). All transformants, transcipients, or transconjugants were streaked for single colony isolates before further examination.

Media, preparation of crude transforming DNA lysates, genetic transformation and $CaCl_2$ transformation, and standard conjugation crosses have been described before (17, 18). Conjugation crosses (by filtration) were considered efficient if they yielded between 10^{-3} and 10^{-1} transconjugants per donor cell used. Inefficient crosses gave frequencies of less than 10^{-4}.

Establishment of the presence of plasmids. The presence of plasmids was established as described by Guerry and co-workers (8, 13) without the lysozyme treatment. DNA so isolated is referred to as ethanol-precipitated DNA. Subsequent agarose gel electrophoresis was performed as reported in reference 17. I used 1% gels (40 mM Tris acetate, 1 mM EDTA, 0.5 μg of ethidium bromide per ml, pH 8) and ran the gels at 15 mA (about 50 V) for 15 h. Chromosomal DNA had then run some 25 to 30 mm. All large plasmids in this study had run about half this distance. Plasmids were considered small if they ran faster than the chromosomal DNA.

Mixed-colony crosses. Two differently genetically marked 15-h old cultures (2 ml of supplemented brain heart infusion; 38°C) were mixed 1:1 and about 1 μl (2×10^6 cells) was spotted on the surface of an agar plate. After 20 h at 38°C all five mixed colonies were suspended in 3 ml of broth and plated in discriminating agar (17) for the observation of transcipients. Recovery of the parents ranged from 6×10^9 to 20×10^9 colony-forming units. In the initial experiments, I added 10 μg of pancreatic DNase per ml (Worthington Laboratories) to the suspensions before mixing. Since no effect was observed, this was not done in later crosses.

RESULTS

Transfer of resistance by genetic transformation. Crude DNA lysates from all original isolates listed in Table 1 were mixed with competent Rd cells. Transfer of resistance was observed for all DNA preparations with the exceptions of NT1056, NT1262, NT1737, NT1760, NT1862, NT1863, and NT1865. Three more independent attempts made with freshly prepared DNA from these strains were unsuccessful. The observed transfer frequencies are listed in Table 1 as transformants per 2×10^8 cells plated. One transformant of each cross was examined for the presence of large plasmids. No plasmid was detected in any of them. Since the transformation frequencies seen were so low, the negative results do not necessarily mean that transfer by genetic transformation is in those cases impossible. Bendler (3) was the first to report this kind of resistance transfer.

Similar DNA preparations from the Rd transformants were now examined for resistance transfer by genetic transformation into *rec*$^+$ and *recA1* recipients. As controls I used DNAs from Rd strains carrying the large plasmids pUB701 (6), pRI234 (11), or pJS1261 (see below). In Table 2 are listed the results of a set of crosses done in 1 day. It is pointed out that the Rd *recA1* strains 1812, 1809, and 1802 are transcipients from mixed-colony crosses with Rd 1316 Amp^r (ampicillin; from Sb1057), Rd 1318 Amp^r (from Sb1245), and Rd 1322 Tet^r (tetracycline; from NT1261), respectively. These *recA1* transcipients all possess a large plasmid, and all are efficient conjugation donors for their resistance in isogenic crosses. As can be seen in Table 2 considerable transfer of resistance with DNA

TABLE 1. *Original antibiotic-resistant Sb and NT H. influenzae and H. parainfluenzae isolates without detectable large plasmids*

Strain	Phenotype	Source	Original designation	Resistance transfer by:		Plasmid	Reference
				Transformation to Rd[a]	Transfer in mixed Rd colony		
NT1056	Amp^r	C. Thornsberry	74-84272	None	No		17
Sb1057	Amp^r	C. Thornsberry	74-64148	100	Yes	pJS1057-2	3, 17
Sb1245	Amp^r	S. Sell	Solomon	90	Yes	pJS1245	17
NT1261	Tet^r	J. Williams	H76	250	Yes	pJS1261	17, 19
NT1262	Tet^r	J. Williams	30270	None	Yes	pJS1262	17, 19
Sb1497	Amp^r	C. Thornsberry	76-46559	110	Yes	pJS1497	17
Sb1503	Tet^r	D. Hansman	H6653/73	2	Yes	pJS1503	9, 17
Sb1728	Tet^r	C. Thornsberry	75-83179	9	Yes	pJS1728	17
Sb1730	Tet^r	C. Thornsberry	76-64886	11	Yes	pJS1730	17
Sb1731	Tet^r	C. Thornsberry	76-53376	10	Yes	pJS1731	17
Sb1732	Tet^r	C. Thornsberry	76-51078	2	Yes	pJS1732	17
Sb1733	Tet^r	C. Thornsberry	76-42556	4	Yes	pJS1733	17
NT1734	Tet^r	C. Thornsberry	76-26540	7	Yes	pJS1734	17
Sb1735	Tet^r	C. Thornsberry	75-72809	1	Yes	pJS1735	17
NT1736	Tet^r	C. Thornsberry	77-00649	1	Yes	pJS1736	17
NT1737	Cam^r	C. Thornsberry	76-81739	None	No		17
Sb1738	Tet^r	C. Thornsberry	75-36325	34	Yes	pJS1738	17
NT1760	Amp^r	J. Saunders	UB2810	None	Yes	pJS1760	15, 17
Sb1842	Amp^r	J. Gray	Pitcher	15	Yes	pJS1842	
NT1862	Amp^r	M. Peel	A2203	None	No		
NT1863	Amp^r	M. Peel	012852	None	No		
Sb1864	Amp^r	M. Peel	014436	6	Yes	pJS1864	
NT1865	Amp^r	M. Peel	921134	None	No		
H. parainfluenzae 1662	Amp^r Tet^r	F. Goldstein	phil	None	No		7
H. parainfluenzae 1761	Cam^r Tet^r	J. Saunders	UB2832	800[b]	Yes[b]	?	15

[a] Transformants per 10^8 cells plated.
[b] Transfer to *H. parainfluenzae* 700 (Boss).

TABLE 2. *Isogenic resistance transfer by genetic transformation with crude DNA lysates from Rd strains with integrated or with free large plasmids*

Donor strain	Plasmid	State	Rd recipient	
			rec$^+$	*recA1*
1316	pJS1057-2	Integrated	1,400[a]	None[b]
1812	pJS1057-2	Free	12	4
1318	pJS1245	Integrated	2,200	None
1809	pJS1245	Free	8	20
1322	pJS1261	Integrated	1,200	None
1802	pJS1261	Free	6	None
1371	pRI234	Free	45	10
1726	pUB701	Free	20	6
1821[c]	pUB702 small	Free	650	550

[a] Transformants per 2×10^8 cells plated.
[b] Less than 2.
[c] The chromosomal Str^r marker transfer was 2.2×10^6.

from donor cultures without detectable plasmids was established only for *rec*$^+$ recipients. It was never seen with *recA1* recipients in the many crosses made. On the other hand, transforming DNA from cultures with free plasmids was biologically active with both *rec*$^+$ and *recA1* recipients. And in the latter ones, all transformants analyzed (one for each cross) carried a large plasmid. Large plasmids can thus be transferred by genetic transformation. Since direct physical evidence for the chromosomal location of the antibiotic resistance genes in strains Rd1316 and 1322 (see Table 2) was given in a previous article (17; also see reference 3) I tentatively concluded therefore that all resistant Rd transformants behaving like the above two strains carry chromosomally integrated resistance genes (Table 1, column 5).

Transfer of resistance of *H. parainfluenzae*. Crude DNA lysates from strain 1761 transformed strain 700 to either Cam^r (chloramphenicol) or Tet^r but never to both. Cam^r transformants (10^{-7} to 10^{-6}) were up to 10 times more frequent. Transformants did not carry a large plasmid. DNA from strain 1662 did not give transformants with strain 700. Neither DNA could transform *H. influenzae* Rd.

Resistance transfer by $CaCl_2$ transformation. Many attempts to transfer established chromosomal markers (such as resistance against streptomycin, novobiocin, or nalidixic acid) into $CaCl_2$-treated Rd bacteria have always failed in my laboratory. It thus appears that chromosomal markers cannot be transferred by this technique.

When $CaCl_2$-treated Rd cells were mixed with ethanol-precipitated DNA preparations from the original isolates, no transformants were seen. The exception was DNA from NT1760 which gave two transformants (out of 10^8 recipient cells plated). Neither transformant carried a large plasmid; this is at present not understood. Control DNA preparations from strains with a large plasmid such as pUB701 (6) and pRI234 (11) gave transformation frequencies of about 10^{-7}, while similar preparations from strains with the small plasmids RSF0885 (5) and pUB702 (15) gave frequencies of up to 10^{-5} (data not shown). DNA from strains Sb1057, NT1760, and *H. parainfluenzae* 1662 and 1761, which possess small plasmids, gave no resistant transformants; hence these small plasmids are considered to be cryptic. The general and consistent failure to transfer antibiotic resistance in this way with ethanol-precipitated DNA from cultures without detectable large plasmids thus supports the view that these strains carry chromosomally integrated resistance genes.

Transfer of resistance within a mixed colony. The original observation of resistance transfer in a mixed colony involved the Tet^r Rd transformant strain 1322 which had been obtained with transforming DNA from the original isolate NT1261. The observed transfer frequency within the colonies was about 10^{-7} per recipient cell recovered. To find out whether this transfer was genetic transformation, strain Rd1322 was mixed with both *rec*$^+$ and *recA1* recipients. DNase at 10 μg/ml was mixed in the cultures in a parallel run (this was eventually left out). The same transfer frequencies were seen for all crosses, i.e., around 10^{-7}. These results thus rule out transfer by genetic transformation. When the transcipients were examined for the presence of a large plasmid, only the *recA1* ones proved to carry one. When the transcipients were examined for conjugational resistance transfer in the standard conjugation cross, again only the *recA1* ones gave efficient transfer. This suggested that the transfer observed involved conjugative plasmid transfer.

Preliminary crosses with all original isolates were mostly negative. I therefore used Rd transformants when available. Most of these gave resistance transfer at frequencies between 10^{-9} and 10^{-7} (see Table 1) with both *rec*$^+$ and *recA1* recipients. When the *rec*$^+$ transcipients (one from each cross) were examined, it was found that all but one carried little or no plasmid, i.e., a very faint plasmid DNA band in the agarose gels might be seen. All but one *recA1* transcipient possessed readily detectable large plasmids. Consistent with these observations were the re-

sults obtained in standard conjugation crosses with the transcipients as donors. Those without detectable plasmids gave few transconjugants (less than 10^{-5}), whereas the *recA1* ones were efficient donors. All these observations suggest strongly that the resistance transfer actually involved transfer of a large plasmid which is not detectable in the primary donor.

Since no Rd transformants had been obtained for a number of original isolates mixed-colony crosses were again carried out with Rd, NT (Reid), and NT (960) as recipients. Strain NT1262 gave resistance transfer to all three recipients, and in all transconjugants examined I found a large plasmid. Repeated crosses with the other donors failed.

Of the two *H. parainfluenzae* strains only 1761 gave resistance transfer to 700 (Boss) in mixed-colony crosses at frequencies of 10^{-8} to 10^{-7} for Tet^r and about 10 times lower for Cam^r. Simultaneous transfer was never seen. All transcipients analyzed did not carry a large plasmid. Unfortunately, no *recA* mutant was available as a recipient. When transcipient clones were used as donors somewhat higher transfer frequencies were sometimes observed (around 10^{-6}). Transfer to strain Rd *recA1* was repeatedly attempted but was never obtained.

Influence of donor *recA1* genotype. The above results suggest that resistance transfer in mixed-colony crosses is due to the transfer of conjugative R plasmids by a very few donor cells (see Discussion). This plasmid is believed to be chromosomally integrated in virtually all resistant cells of the culture except for a few bacteria in which it had been excised. To find out whether this excision is *recA*$^+$ gene product-dependent, the Tet^r Rd transformant 1322 (from NT1261) was transformed to *recA1*. Two clones were tested for sensitivity to methyl methane sulfonate and for transformability. Neither clone carried a large plasmid. Both *recA1* as well as two *rec*$^+$ Rd 1322 subclones transferred Tet^r normally (10^{-7}) in mixed-colony crosses with another Rd recipient. One subclone of each of the four clones was again tested, and the same results were obtained. This indicates that if excision of integrated plasmids can occur, it is independent of the *recA*$^+$ gene product.

Re-integration of transferred plasmid. Transfer of resistance to *recA1* recipients within a colony virtually always correlated with transfer of a large conjugative R plasmid. Transfer of resistance to *rec*$^+$ recipients, on the other hand, often gave transcipients without such large plasmids. Since all transcipient cultures were always grown in media with antibiotics, loss of plasmid cannot be the explanation. The difference in behavior between *rec*$^+$ and *recA1* recipients may be understood by assuming that *rec*$^+$ transconjugants frequently and quickly integrate the transferred plasmid, whereas *recA1* recipients rarely do so. Most of the integration (in *rec*$^+$ recipients) may occur through regions of homology between plasmid and chromosome which, as generalized recombination, should not occur in *recA1* recipients. Less frequent integration may occur through site-specific recombination which is *recA*$^+$ independent. This explanation has been offered for similar observations of integration of the temperate phages P1 and P7 into the *Escherichia coli* chromosome (4).

DISCUSSION

The key observations in this study are as follows. (i) No large plasmids were detectable in 25 antibiotic-resistant *Haemophilus* isolates. (ii) Resistance of at least 16 of these strains could be transferred to strain Rd *rec*$^+$, but not to Rd *recA1*, by genetic transformation. (iii) Ethanol-precipitated DNA did not transform $CaCl_2$-treated Rd recipients (one exception), whereas similar DNA from large plasmid-carrying strains did. (iv) Rd genetic transformants did not possess large plasmids. (v) These transformants transferred resistance to both *rec*$^+$ and *recA1* recipients in mixed-colony crosses. (vi) The resultant *recA1* transcipients carried large plasmids and were efficient donors of the acquired resistance in subsequent standard conjugation crosses. The most plausible explanation of these observations is that the large majority of the original isolates and their resistant Rd transformants carried an integrated conjugative R plasmid. This integrated plasmid is always excised by a few cells in every population, and it is these cells which transfer resistance by conjugation to recipients when mixed with them on an agar surface. This excision is not controlled by the *recA*$^+$ gene. An alternative explanation which assumes that resistance transfer involves plasmid-mobilized chromosome transfer, followed by integration into the recipient chromosome, is not likely since *recA1* Rd cells are efficient recipients.

The observed transfer frequencies in mixed-colony crosses may not reflect actual transfer per donor cell. Model experiments with donor-recipient ratios of much less than one have shown that plasmids can spread rapidly through such a population (manuscript in preparation). Thus, the actual frequencies are probably much lower, and this may be the reason why many of the donors scored negative in earlier standard conjugation crosses (17).

Bendler, who was the first to report resistance

transfer by genetic transformation (3), speculated that this involved the addition to the recipient's genome of a donor DNA segment carrying the Amp^r genes. He mentions that this segment could be an integrated plasmid as described by Elwell et al. (5) and calculated it to be between 22×10^6 and 30×10^6 daltons. One of the two strains studied by him was Sb1057 (which carries two small cryptic plasmids). Indeed, *Eco*RI digestion of the excised plasmid in Sb1057 (isolated from a *recA1* transconjugant) showed that it is identical or very similar to Elwell's plasmid RSF007 (reference 5; manuscript in preparation). Thus, one can envisage the transformation process as an instance of synapsis between donor DNA regions flanking the integrated plasmid and similar homologous regions in the recipient DNA. By postulating two crossovers on either side of the plasmid, integration of this plasmid into the recipient genome can be understood. It is clear, then, why this process does not occur in *recA1* recipients since the *recA*$^+$ gene product controls generalized recombination and presumably synapsis. Perhaps the exceptional NT isolates which did not give resistance transfer by genetic transformation carry their integrated plasmids in DNA regions with no homology to any DNA region in the Rd recipient.

My results provide an answer to a question asked in a previous report (17) as to why so many antibiotic-resistant *H. influenzae* isolates appear to carry chromosomally located resistance genes. As I speculated, cells in nature become resistant by receiving a conjugative plasmid. Most of them eventually integrate the entire plasmid into their chromosomes. This appears to rule out my speculation that translocation of the resistance transposon from plasmid to chromosome had taken place.

Three questions now need to be answered. (i) What is the genetic organization of the resistance genes in the exceptional isolates which never transferred them in any way? These strains might carry integrated nonconjugative plasmids. Such plasmids have never been observed to transfer in mixed-colony crosses (manuscript in preparation). (ii) How much are all these plasmids related? Do they have a common ancestor, as has been suggested by Harkness and Murray for Ap plasmids (10)? My preliminary experiments with *Eco*RI have shown that most of the Ap plasmids (column 7, Table 1) have very similar or identical enzyme digests, but this was not seen for Tc plasmids (manuscript in preparation). (iii) Is the conclusion made earlier (17) still valid that recombination can occur between integrated plasmid (i.e., chromosome) and a (different) free plasmid? Again, *Eco*RI digestion of the so-called recombinant plasmids described in reference 17 has shown that this conclusion may be in error.

It can now be understood why some fresh hospital isolates of *H. influenzae* may quickly loose the ability to transfer their antibiotic resistance by conjugation (see reference 15). The freshly isolated culture may still contain enough cells with free plasmid available for transfer. Upon subculturing in the laboratory, most or nearly all cells integrate the plasmid into the chromosome, from where it cannot be transferred by conjugation.

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