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Comparative Analysis of *Enterococcus faecalis* Sex Pheromone Plasmids Identifies a Single Homologous DNA Region Which Codes for Aggregation Substance

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An analysis of the 11 known sex pheromone plasmids of *Enterococcus faecalis* was performed by DNA-DNA hybridization. Plasmids pAD1, pJH2, and pBEM10 turned out to be closely related, whereas pAM373 showed only weak homology with pAD1. A comparison of the hemolysin/bacteriocin determinants of pAD1, pJH2, and pOB1 revealed strong similarities at the DNA level. Our main finding was that one DNA region is conserved among all sex pheromone plasmids, with pAM373 again being an exception; for pAD1 this region was shown earlier to code for aggregation substance. Detailed hybridization studies of the genes for this plasmid-coded adhesin, which is responsible for cell-cell contact during conjugative transfer via the so-called sex pheromone system of *E. faecalis*, support the idea of their common origin.

A group of conjugative plasmids of *Enterococcus faecalis* which transfers at high frequencies (10^{-3} to 10^{-1}) in broth matings has been described. Mating is triggered by sex pheromones (small, linear peptides) excreted into the medium by plasmid-free cells. These sex pheromones induce the synthesis of an adhesin called aggregation substance which appears on the surface of cells carrying the corresponding plasmids (sex pheromone plasmids [for reviews, see references 5 and 8]).

Mature aggregation substance coded by the sex pheromone plasmid pAD1 is a protein of 137 kDa that appears as a dense layer of hairlike structures on the cell surface (14, 26). The adhesin enables the contact of donor and recipient which is necessary for the transfer of a copy of the plasmid. Once a cell has acquired the plasmid it shuts down the excretion of active sex pheromone corresponding to the pAD1 plasmid but still excretes unrelated pheromones (9).

To date 11 plasmids which are known to encode a pheromone response have been described: pAM373, pAD1, pAMγ2, pAMγ3, pJH2, pPD1, pCF10, pOB1, pBEM10, pAM323, and pAM324 (5). Another plasmid, pAMγ1, turned out to be very similar if not identical to plasmid pAD1 (6; unpublished data); both plasmids respond to the same sex pheromone and are incompatible, and different restriction enzymes, even if used in combination, resulted in DNA fragments of identical size. With the exception of pAM373 all these plasmids have similar sizes, which range from 54 to 71 kb (Table 1). Only a few phenotypic markers for them have been identified: antibiotic resistances (Pn^r, Gm^r, Km^r, and Tm^r for pBEM10; Em^r for pAM323; and the Tc^r for pCF10, which is due to a Tn*925* insertion [1]), a hemolysin/bacteriocin determinant (for pAD1, pJH2, and pOB1), and a bacteriocin determinant (for pPD1 and pAMγ2). Immunological cross-reactivity of plasmid-coded surface proteins expressed after induction with sex pheromone already indicated a similarity between some of these plasmids for the genes involved in conjugal transfer (11, 28). A possible problem with these earlier studies stems from the fact that antibodies against whole, induced cells were used. Only for pAD1- and pPD1-carrying strains was the comparison made with proteins separated by gel electrophoresis and Western immunoblotting (11). The other comparative study (28) used whole cells, and therefore an observed immunological cross-reactivity is not necessarily due to similar aggregation substances (induction results in the synthesis of more than one surface protein [11]). Here we present data on a systematic comparison of all sex pheromone plasmids at the DNA level; as a reference plasmid we have chosen pAD1, the best-studied sex pheromone plasmid.

Overall homology of all 11 sex pheromone plasmids. The 11 sex pheromone plasmids were first compared with total plasmid DNA of pAD1. For isolation of plasmid DNA, *E. faecalis* strains were grown in 1 liter of antibiotic medium no. 3 (AB3; Oxoid Ltd., Wesel, Federal Republic of Germany), and the method of Somkuti and Steinberg (24) was used for DNA preparation. The DNA was further purified by CsCl buoyant equilibrium density centrifugation. Since plasmids pAMγ2 and pAMγ3 are associated with a second plasmid, pAMα1 (Table 1), which shows some homologies to pAD1 (unpublished data), pAMγ2 and pAMγ3 had to be separated from pAMα1 by agarose gel electrophoresis and were isolated from the gel by use of Glassmilk (Geneclean kit; Bio 101, Inc., La Jolla, Calif.).

The DNA of the 11 plasmids was blotted onto nylon membranes in serial dilution steps from 200 to 1.5 ng per well with a slot blot unit. The bound DNA was probed with nick-translated pAD1 DNA which had been digested with *Eco*RI prior to radioactive labeling. After autoradiography, densitometric readings were used to calculate the degree of homology of the different plasmids to pAD1 (the homologous hybridization of pAD1 was referred to as 100%) (see Table 1 for experimental details). The results obtained show very clearly that pJH2 and pBEM10 share extensive DNA-DNA homology with pAD1, whereas pAM373, pAMγ2, pPD1, and pAM324 exhibit only weak homology.

Comparison of the hemolysin/bacteriocin determinants of pAD1, pJH2, and pOB1. An earlier comparison of hemolytic isolates of *E. faecalis* from different geographical areas had shown that plasmid-encoded hemolysin and bacteriocin ac-

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TABLE 1. Bacterial strains and corresponding plasmids known to encode a pheromone response

Host strain	Plasmid (size [kb])[a]	Phenotype	Source or reference	Homology[b]
FA2-2	pAM373 (37)		2	15
DS16C2	pAD1 (58)	Hly/Bac Uv^r	12	100
JH2-2	pAMγ2 (54)	Bac	6	22
	pAMα1 (9)	Tc^r		
JH2SS	pAMγ3 (54)		6	40
	pAMα1 (9)	Tc^r		
OG1S	pJH2 (58)	Hly/Bac	[c]	95
OG1X	pPD1 (56)	Bac Uv^r	[c]	18
OG1SSp	pCF10 (65)	Tc^r (Tn*925*)	10	35
OG1RF	pOB1 (69)	Hly/Bac	[c]	60
OG1X	pBEM10 (71)	Pn^r Gm^r Km^r Tm^r	22	85
OG1X	pAM323 (63)	Em^r	22	60
OG1X	pAM324 (56)		22	20

[a] Sizes of the plasmids were determined by summing up the sizes of all fragments observed on an agarose gel after *Eco*RI digestion; we note slight differences from the sizes cited in the references.

[b] Quantitative nucleic acid hybridizations were performed with a slot blot unit. Densitometer readings were carried out with a spectrophotometer (Gilford System 2600) running the predefined distance scan routines at a wavelength of 550 nm. The maximum absorbance peak was determined for all dilutions of each plasmid, and peak areas were calculated. These data were plotted versus the corresponding DNA concentration in the range in which they were linear. The slopes of all these lines, each representing a plasmid, compared with that of pAD1 define the degree of homology, which is expressed in percentage (with pAD1 set as 100%). The values were corrected for the different plasmid sizes.

[c] These plasmids were transformed into the corresponding strains in the lab of Don B. Clewell.

tivities are often associated and that these hemolysin plasmids are closely related (20). Ike et al. (18) studied the hemolysin/bacteriocin of pAD1 in detail and referred to it as a cytolysin because of its lytic properties on prokaryotic and eukaryotic cells. This cytolysin consists of two components, A and L; component A (probably a serine protease) activates component L, the lysin, and contributes to the immunity of the cell against its own lysin. The gene cluster responsible for the expression of components A (*cylA*) and L (*cylL*) and for the export of the cytolysin (*cylB*) spans a region of approximately 7 kb (15). The earlier study (20) used for detection of the cytolysin a *Bam*HI-*Sal*I fragment which covers the second half of component L and all of component A and extends for more than 1 kb over the end of the *cylA* gene.

Restriction fragments were separated in 0.7% agarose gels in TAE buffer (0.04 M Tris-acetate, 0.001 EDTA [pH 7.8]) as previously described (21). DNA was transferred from agarose gels to a nylon membrane (Hybond-N^+, Amersham-Buchler) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.2]) by the method of Southern (25). Hybridization probes were radiolabeled with [α-^{32}P]dATP as previously described (21). Labeled probe was separated from unincorporated [α-^{32}P]dATP with an ELUTIP-d column (Schleicher & Schuell). Hybridization at high stringency was performed in 0.6× SSC and 0.7% dried skim milk at 65°C for probe 1 and 60°C for probe 2 (see text) for 12 to 16 h, which was followed by two washes (30 min each) in 2× SSC and 0.1% sodium dodecyl sulfate (SDS) containing 0.25% dried skim milk at room temperature and a final wash (30 min) at 70°C in 0.1× SSC and 0.1% SDS. Bound probe was visualized by autoradiography. For rehybridization experiments the membrane was washed for 15 min in 0.1× SSC and 0.1% SDS at 100°C.

To test for the presence of *cyl*-related genes, the 11 sex pheromone plasmids were digested with *Eco*RI and the DNA fragments were separated by agarose gel electrophoresis (Fig. 1A) and transferred to a nylon membrane. Hybridization was carried out by using two different probes of the DNA region coding for the cytolysin of pAD1. Probe 1 (which consisted of an *Eco*RV-*Eco*RI fragment of *Eco*RI fragment C) represents the first one-third of component L; probe 2 covers the rest of component L and all of component A (probe 2 was composed of the *Eco*RI fragments F and H and an *Eco*RI-*Pst*I fragment of *Eco*RI fragment D [see reference 18 for the respective restriction maps]). As expected, pAD1 (the positive control) showed hybridization signals for *Eco*RI fragments C, D, F, and H (Fig. 1B and C, lanes b). In the case of pJH2 (lanes e), probes 1 and 2 revealed strong homologies to the pAD1-encoded cytolysin (Fig. 1B and C, respectively). For pOB1 (lanes h), probe 1 showed no homology (Fig. 1B), while probe 2, which covers the *cylL*, *-B*, and *-A* genes, again indicated strong homology (Fig. 1C). The hybridization signal detected with probe 1 for pAM373 and pBEM10 (Fig. 1B, lane a and i) could be due to the DNA region (approximately 140 bp of which is covered by probe 1) upstream of an open reading frame which probably is required for expression of component L (16). Plasmids pAMγ2 and pPD1, both coding for bacteriocin activity alone, showed no similarity to the bacteriocin/hemolysin determinant of pAD1.

Hybridization studies with different probes of the gene encoding aggregation substance on pAD1. Sex pheromone plasmids were digested with *Eco*RI, and the fragments were blotted onto a nylon membrane after separation on an agarose gel. By probing consecutively with all plasmids after ^{32}P labeling, we identified one DNA region which shows strong similarity among all sex pheromone plasmids (except for pAM373) and covers the region of pAD1 coding for aggregation substance (data not presented). Degenerated oligonucleotides which were deduced from the N-terminal sequence of pAD1-encoded aggregation substance and had been used for localization of the gene on pAD1 (13) also hybridized with most of the other sex pheromone plasmids, again indicating similarities in this region (data not shown). Indeed, DNA sequence data for the plasmids pAD1 and pCF10 strongly support this view (see below).

The gene coding for aggregation substance of pAD1 (*asa1*) has been sequenced recently (13). It spans the *Eco*RI fragments E, I, and A on the pAD1 physical map over a total length of more than 4 kb. Hybridization experiments were carried out with four different DNA probes covering the complete *asa1* gene (Fig. 2). For these hybridizations, the same nylon membrane, with *Eco*RI fragments of all 11 plasmids, was used as for the comparison of the hemolysin/bacteriocin determinant (Fig. 1).

Lanes b of the four resulting autoradiographs (Fig. 2B) correspond to the positive control, namely, the homologous hybridization with pAD1. As expected, probe 1 hybridized with fragment E and probe 2 hybridized with fragment I, with fragment A, and very faintly with fragment E, whereas probes 3 and 4 showed a strong signal with fragment A (Fig. 2 [see references 3, 4, and 13 for a detailed restriction map of pAD1]). No homology could be observed for pAM373 (lanes a). With the exception of pAM324 (panel 1, lane k) showing no hybridization signal with probe 1, all other plasmids showed a more or less extensive homology with the entire DNA region coding for the adhesin. In most cases the genes for aggregation substance span two or three *Eco*RI fragments; only for pAM324 does one single fragment hybridize

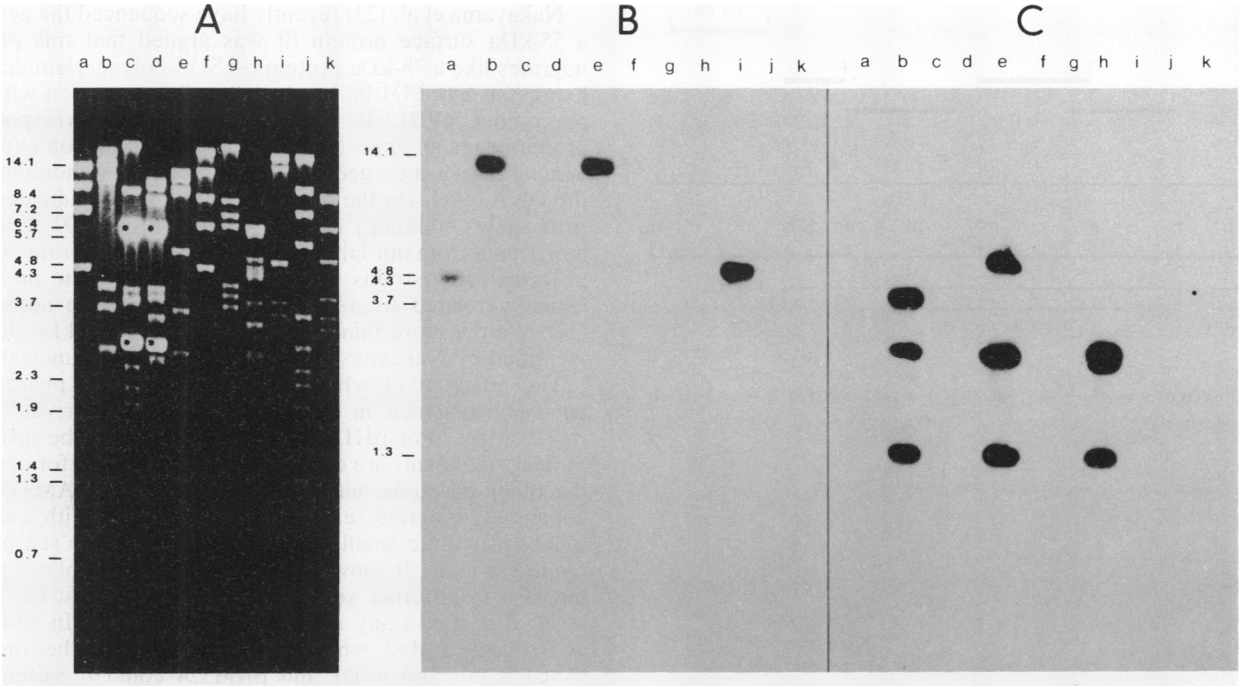

FIG. 1. *Eco*RI restriction patterns of the 11 sex pheromone plasmids and Southern hybridizations with ^{32}P-labeled probes of the hemolysin/bacteriocin-coding region of pAD1. (A) Agarose gel electrophoresis of *Eco*RI-digested pAM373 (lane a), pAD1 (lane b), pAMγ2 (lane c), pAMγ3 (lane d), pJH2 (lane e), pPD1 (lane f), pCF10 (lane g), pOB1 (lane h), pBEM10 (lane i), pAM323 (lane j), and pAM324 (lane k). The molecular sizes (in kilobases) refer to λ DNA digested with *Bst*EII. The fragments in lane c and d indicated by asterisks represent the two *Eco*RI fragments of pAMα1, a plasmid associated with pAMγ2 and pAMγ3. (B and C) Autoradiographs showing hybridization with probe 1 (B), an *Eco*RV-*Eco*RI fragment of *Eco*RI fragment C of pAD1, and probe 2 (C), consisting of *Eco*RI fragments F, H, and the *Eco*RI-*Pst*I part of *Eco*RI fragment D.

with three different probes. The sizes of the hybridizing *Eco*RI fragments and their degrees of intensity indicate that the pAD1-, pJH2-, pBEM10-, and pAM323-encoded adhesins are closely related (lanes b, e, i, and j, respectively). Nevertheless, the location of the *Eco*RI cleavage sites in the genes for aggregation substance seems to be of secondary importance for comparative studies, e.g., pCF10 shows a high degree of identity with pAD1 for their aggregation substance genes, even though the *Eco*RI digestion patterns of both plasmids are different.

Conclusions. The overall DNA homology comparisons of the 11 plasmids revealed a close relationship between pAD1 and pJH2. This is further supported by the following data: (i) pJH2-harboring cells can be induced by synthetic sex pheromone cAD1 (unpublished data); (ii) both plasmids possess a probably identical hemolysin/bacteriocin determinant (this study); and (iii) pAD1 and pJH2 are members of the same incompatibility group designated IncHly by Colmar and Horaud (7).

Although pOB1 shares a homologous cytolysin determinant with pAD1, another nonhemolysin plasmid, namely, pBEM10, is much more related to pAD1. Like pJH2, pBEM10 can be induced with synthetic sex pheromone cAD1 (22).

Our comparative study was performed under stringent hybridization conditions. At a hybridization temperature of 65°C, the signals detected on the corresponding autoradiographs showed an intensity reduced to one-third compared with the bands detected after hybridization at 60°C (data not presented). In the case of the hemolysin/bacteriocin probe 2 we had to use a hybridization temperature of 60°C, because only very weak signals were detected after hybridization at 65°C. This might well be due to the fact that the G+C percentage of this DNA region is very low compared with the rest of the plasmid (24 versus 38% [16]). The data obtained clearly speak for a high conservation of the hemolysin/bacteriocin genes coded by the plasmids pAD1, pJH2, and pOB1; on the other hand, the existence of previously undetected *cyl*-related genes in all known sex pheromone plasmids seems to be excluded by these results.

The main conclusion to be drawn from this study is that there exists strong homology among the sex pheromone plasmids regarding their genes for aggregation substance. A comparison of pAD1 and pCF10 indicated not only a similar organization of sex pheromone-induced genes but especially an identity of more than 85% for the structural genes coding for aggregation substance (27). Significant differences appear only in a region corresponding to *Eco*RI fragment I of pAD1. This is confirmed by our hybridization studies, which show only a very weak signal with probe 2 of *asa1*, while probes 1, 3, and 4 show strong signals (Fig. 2B, lane g). Nucleotide identities for these regions are 32, 91, 95, and 96%, respectively. Interestingly, the degree of homology for the four probes varies for each plasmid. This result and the fact that the probes used for hybridization represent rather large fragments make it impossible to predict conserved regions for all plasmids. The existence of at least one (more or less) conserved binding site should be expected for all sex pheromone plasmid-encoded adhesins, because they mediate binding to the same receptor (probably lipoteichoic acid [11]) on the surface of the recipient. The presence of different specific receptors appears unlikely.

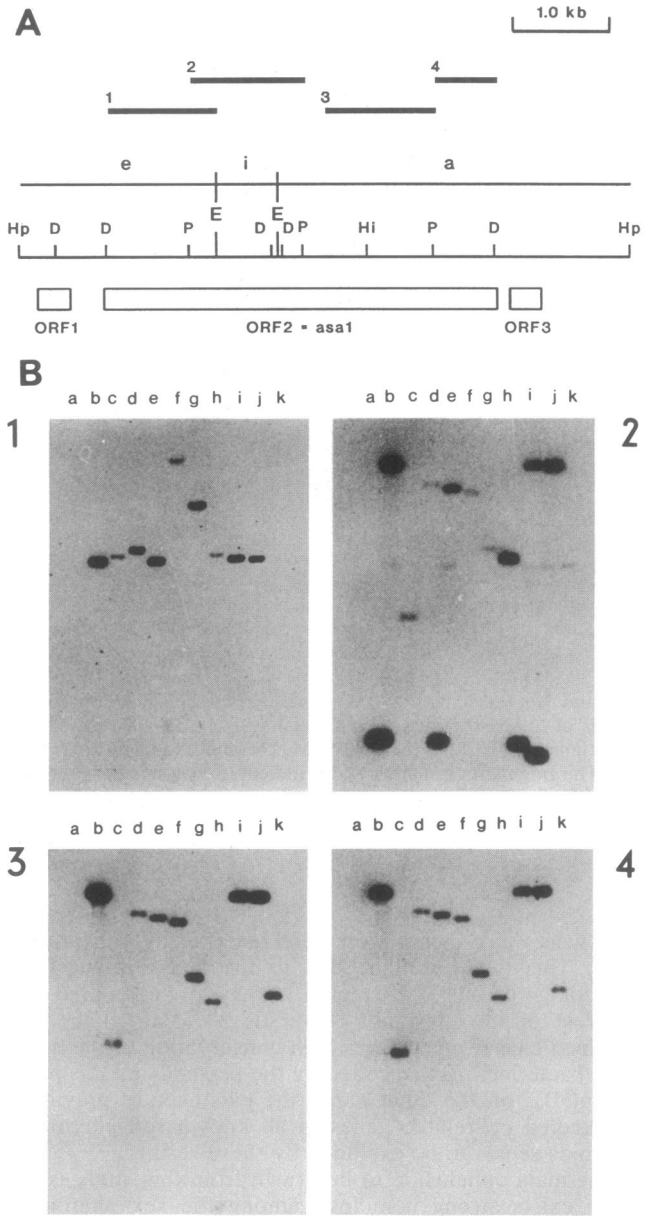

FIG. 2. Comparison of the 11 sex pheromone plasmids for aggregation substance *asa1* homologous genes. (A) DNA region of pAD1 known to encode aggregation substance (*asa1* [13]). The gene is flanked by two open reading frames of unknown function, ORF1 and ORF3. Abbreviations for restriction enzyme cleavage sites are as follows: D, *Dra*I; E, *Eco*RI; Hi, *Hin*dIII; Hp, *Hpa*II; and P, *Pst*I. *Eco*RI fragments E, I, and A of pAD1 are indicated by lowercase letters. The different probes used for hybridizations are indicated as black bars and are numbered 1 to 4. (B) Autoradiographs of the probes shown in panel A (numbered correspondingly). The membrane used in this figure is identical with the one used for Figure 1 and therefore contains *Eco*RI fragments of pAM373 (lanes a), pAD1 (lanes b), pAMγ2 (lanes c), pAMγ3 (lanes d), pJH2 (lanes e), pPD1 (lanes f), pCF10 (lanes g), pOB1 (lanes h), pBEM10 (lanes i), pAM323 (lanes j), and pAM324 (lanes k).

Only pAM373 seems to code for a totally different adhesin, since no homology at all to the *asa1* gene could be detected, at least not under the stringent hybridization conditions used in this study.

Nakayama et al. (23) recently have sequenced the gene for a 55-kDa surface protein (it was argued that this protein migrates like a 78-kDa protein on SDS-polyacrylamide gels) expressed by pPD1-harboring cells after induction with sex pheromone cPD1. It was argued that the corresponding protein plays an important role for the aggregation event. A comparison of their gene and *asa1* revealed no homology at the DNA level. On the other hand, this study indicates that *asa1* shows homology to a certain region of pPD1. Furthermore, data from our laboratory indicate that after induction, pPD1-carrying strains express a 137-kDa protein immunologically related to pAD1-encoded aggregation substance (17). We therefore think that the role of the 78-kDa protein identified by Nakayama et al. (23) in mating is unclear.

The question of whether some of the sex pheromone plasmids analyzed in this study are mere derivatives of pAD1 arises. For pJH2 such a view seems to be justified. Similar statements are currently difficult to make for many of the other plasmids, although it is clear that pAM373 is a completely different sex pheromone plasmid. With a size of 37 kb, it is quite small compared with all other sex pheromone plasmids. It shows weak homology not only to pAD1 but also to all other sex pheromone plasmids at the DNA level, if it shows any at all (data not shown). In addition, pCF10 and pAMγ2, which are related to each other (unpublished data), and pPD1 and pAM324 could represent different groups.

Our data support the idea of a common origin of the sex pheromone-induced mating response. The genes responsible for this special type of conjugation probably have been acquired by different groups of plasmids, resulting in the current existence of 11 sex pheromone plasmids.

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