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Viability During Storage and Stability of Plasmids During Storage and Subculturing in Strains of *Vibrio anguillarum*

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Pedersen, K.: Viability during storage and stability of plasmids during storage and subculturing in stratins of *Vibrio anguillarum.* Acta vet. scand. 1997, 38, 315-322. – The stability of plasmids, 3.3 kb – ~200 kb, in 8 strains of *Vibrio anguillarum* displaying different plasmid profiles and reactions with O-antisera was investigated over an 18 months period. All plasmid profiles proved to be resistant to storage at different temperatures but strains stored at 37°C were only viable for a short period of maximum 2 months. Strains stored at 5°C and 20°C were viable for a longer period. Viable strains maintained their plasmid profile throughout the experiment, except in 2 cases where a 67 kb and a 200 kb plasmid were lost. Strains stored at –80°C all remained viable and maintained their plasmid profile throughout the study. By subcultivating daily for up to 100 successive days, most strains maintained their plasmid profiles. Only 2 strains lost their plasmid. When picking 100 single colonies from agar plates, none of the colonies showed plasmid profiles deviating from the expected. The results suggest that plasmid profiles among *V. anguillarium* are very stable during subculturing, storage and laboratory handling using standard laboratory procedures, and thus, reliable for epidemiological investigations. In a second experiment, 2 pairs of 2 strains were grown together in mixed cultures. They were identical in all traits, except that one strain in each pair harboured the 67 kb pJM1-like virulence plasmid, whereas the other had lost this plasmid. The result showed that the growth rate was the same for strains with and without the plasmid, indicating that under laboratory conditions, this plasmid is neither a benefit nor the opposite for bacterial growth.

Introduction

Vibrio anguillarum is an important marine fish pathogen for farmed as well as feral fish in salt waters, but it is also a bacterial species that plays a role as a member of the natural marine bacterial flora (*Austin & Austin* 1993, *Larsen* 1990). *V. anguillarum* has been shown to exist in several O-serovars of which serogroup O1, O2, and O3 seem to be the most important ones as fish pathogens whereas remaining serogroups are considered to be mainly environmental strains (*Austin et al.* 1995, *Larsen* 1990, *Sørensen & Larsen* 1986). Some strains carry plasmids, although plasmids are more frequent in some O-serogroups than in others (*Austin et al.* 1995, *Olsen & Larsen* 1993, *Pedersen et al.* 1996b). Plasmids in *V. anguillarum* are interesting from several points of view. Thus, some large plasmids have been described – especially from Japan – to carry antibiotic resistance factors (*Aoki et al.* 1974, 1985), and among sero-

group O1 strains, a 67 kb plasmid – or derivatives of it – is important for virulence by encoding an iron-sequestering system (*Crosa* 1980, *Pedersen et al.* 1997b). Plasmid profiles have also been suggested as one of several typing methods and as epidemiological markers in *V. anguillarum* (*Larsen & Olsen* 1991, *Pedersen et al.* 1996b, *Skov et al.* 1995, *Tiainen et al.* 1995). However, in order to use plasmid profiles as a reliable typing method, these profiles should not change during the isolation process and subsequent laboratory handling, including subculturing, storage, and shipment to other laboratories. In a report by *Olsen et al.* (1994), it was found that some plasmids in *Salmonella berta* and *Salmonella enteritidis* were lost during storage, but with a frequency increasing with the temperature from 5 °C to 30 °C. Likewise, in a recent study of atypical *Aeromonas salmonicida,* some variation in plasmid profiles among strains isolated repeatedly from the same outbreak was demonstrated, suggesting instability of plasmids *in vivo* in this species (*Pedersen et al.* 1996a). Similar observations have also recently been reported for *Vibrio damsela* (*Pedersen et al.* 1997a). New information on the pJM1-like 65 – 67 kb virulence plasmid of *V. anguillarum* serogroup O1 indicates that this plasmid is stable *in vivo* and not transferred to other strains (*Pedersen & Larsen* 1995). In contrast, in another study, it was indicated that strains may loose this plasmid spontaneously (*Skov et al.* 1995). Likewise, it has been possible to cure *V. anguillarum* strain 775 from this plasmid (*Crosa et al.* 1980). Information on other plasmids from *V. anguillarum* is not available. The purpose of the present study was to evaluate the stability of various plasmids with different molecular weights from *V. anguillarum* strains displaying different reactions with O-antisera and originating from various sources, during storage and laboratory handling.

Materials and methods

Bacterial strains and culture conditions

Ten strains of *V. anguillarum* were studied (Table 1). Eight strains were used for the plasmid stability investigations (Table 2) and 2 for study of growth in mixed cultures. The strains were selected from the culture collection of the laboratory and kept at –80 °C. When resuscitated from the freezer, they were propagated on blood agar (BA) (marine agar (Difco) supplemented with 5% calf blood) and incubated at 20 °C for 48 h. From the blood agar cultures, the strains were subcultured and stored as described below.

Serotyping

Serotyping on the basis of heat stable O-antigens was carried out as described by *Larsen et al.* (1993), using rabbit antisera against the serogroups O1 – O10 (*Sørensen & Larsen* 1986) and 4 additional serogroups, introduced by *Austin et al.* (1995) and further described by *Pedersen et al.* (1998).

Plasmid profiling

Plasmid DNA was extracted using the method of *Kado & Liu* (1981) and separated by electrophoresis in 0.8% agarose gels in TAE buffer. DNA was visualized by staining with ethidium bromide and photographed at 254 nm UV transillumination whereafter plasmid molecular weights were calculated as described by *Tiainen et al.* (1995).

Examination of plasmid stability in vitro

Three different approaches to evaluate plasmid stability were attempted. First, stability was studied by storage at different temperatures. From blood agar plates, broth cultures were prepared in Luria Bertani broth (LB, Gibco) supplemented with 0.5% (w/vol) NaCl. From each broth culture, a sample was supplemented with 30% – 50% glycerol and stored at –80 °C

and in addition, a series of stab cultures were made in stock culture agar (STC agar, Difco) and stored at 5 °C, 20 °C and at 37 °C, respectively. At intervals, a stab culture, stored at each temperature, was broken and streaked onto BA. Each time a new stab culture was used, and the broken vial was discarded. Simultaneously, material from the vials kept in the freezer at –80 °C was streaked on BA. From BA, a single colony culture of each strain was prepared in LB for plasmid profiling. On one occasion, 10 single colonies instead of one were collected from each of the still viable cultures and propagated separately in LB for profiling. In a second experiment, strains were subcultured daily in LB for 100 successive days by transferring one loopful of overnight broth culture to 5 ml of fresh LB medium followed by incubation overnight at 20 °C. Once every week, the cultures were examined for plasmid content and streaked on BA for control of purity and identity with the original strains. In the third experiment, material from an overnight broth culture was streaked onto BA and incubated for 2 days at 20 °C. Then, 100 single colonies were picked and each transferred to a tube with LB, incubated overnight at 20 °C and studied for plasmid contents.

Mixed cultures

The 2 strains, 87-9-116 and 87-9-117 (Table 1), were used in a study of mixed cultures. These 2 strains had been isolated from the same outbreak and were identical in all traits, except that 87-9-116 carried the pJM1-like virulence plasmid, whereas 87-9-117 had no plasmids. Likewise, the pJM1-containing strain VIB 1 and the plasmid-free derivative of it, obtained in the present study by subculturing, were examined in mixed cultures. By dilution and spreading on tryptone soya agar (Oxoid) supplemented with 0.5% NaCl, it was found that overnight broth cultures of each of the 2 pairs of strains had the same concentration of bacteria. Broth cultures of both pairs of strains were therefore prepared whereafter 5 ml LB were inoculated with 100 µl broth culture of each strain, one broth culture for each pair of strains, and incubated at 20 °C overnight. Then, one loopful of the mixed culture was streaked on BA and incubated at 20 °C for 2 days. Eighty and 48 single colonies, respectively, from each pair of strains were picked and analysed for plasmid content.

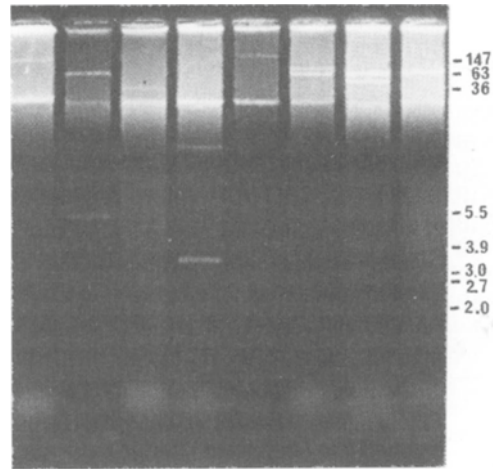

Figure 1. Plasmid profiles of the *Vibrio anguillarum* strains. Lane 1: VIB 225, 147 kb; lane 2: VIB 243, 5.3 and 67 kb; lane 3: VIB 207, 36 kb; lane 4: VIB 188, 3.3 and 12.3 kb; lane 5: VIB 103, ~200 kb; lane 6: VIB 56, 67 and 84 kb; lane 7: VIB 18, 50 and 67 kb; lane 8: VIB 1, 67 kb. Molecular weight markers are indicated to the right.

Results

The plasmid profiles of the *V. anguillarum* strains are shown in Fig. 1 and the size of the plasmids is reported in Table 1.

Storage experiments

Most cultures maintained their plasmid profile during storage. Plasmids were lost only in 2 cases: VIB 103 stored at 5 °C and VIB 243

Table 1. *Vibrio anguillarum* strains used in a study of plasmid stability.

Strain	Source and origin	O-serotype	plasmid content, kb
VIB 1[1]	Rainbow trout, Denmark	O1	67
VIB 18[1]	Rainbow trout, Denmark	O1	50, 67
VIB 56[1]	Sea bass, Italy	O1	67, 84
VIB 103[1]	Cod, Denmark	O2	~200
VIB 188[1]	Sea bream, Spain	Cross reacting	3.3, 12.3
VIB 207[1]	Rotifers, Greece	Non-typeable	36
VIB 225[1]	Water, Greece	Non-typeable	147
VIB 243[1]	Sockeye salmon, USA	O1	5.3, 67
87-9-116[2]	Rainbow trout, Finland	O1	67
87-9-117[2]	Rainbow trout, Finland	O1	0

[1] Strains are described in more detail by Austin et al. (1995).
[2] Strains are described in more detail by Skov et al. (1995).

stored at 37 °C had lost a 200 and a 67 kb plasmid, respectively, when screened after 1 month of storage (Table 2). However, most of the strains did not remain viable at certain temperatures during the whole experiment. When stored at –80 °C, all strains remained viable during the whole period of 18 months, and they always displayed the same plasmid profile. When stored at 37 °C for one month, two strains had a very weak growth in broth, and one strain grew almost normally, but had lost one of its 2 plasmids. The remaining cultures were dead. After 2 months, no cultures kept at 37 °C were viable. When stored at 5 °C or 20 °C, most strains remained viable for several months, but only 2 strains remained viable during the whole period (Table 2). Some cultures could display a very weak growth in broth after resuscitation (Table 2). In these cases, plasmids were never detected. On one occation, when 10 single colonies from each of the resuscitated cultures were tested instead of just one colony, these 10 colonies all had the same plasmid profile.

Subculturing experiments

In the subculturing experiments, loss of plasmids or change in plasmid profile were recorded for 2 of the strains. Strain VIB 1 lost its 67 kb plasmid after 73 subculturings, and after 31 subculturings, VIB 225 had lost its large 147 kb plasmid.

Mixed cultures

When growing the 2 strains, 87-9-116 and 87-9-117, with and without the pJM1 plasmid, respectively, together, and examining 80 colonies for plasmid content, 39 colonies carried the plasmid and 41 colonies had no plasmid ($\chi^2 = 0.05$). Likewise, when VIB 1 was cultured together with a cured VIB 1 derivative, 27 of 48 colonies carried the plasmid, whereas 21 were plasmid-free ($\chi^2 = 0.75$).

Discussion

Storage experiments

It is well known that bacteria may loose their plasmids at elevated temperatures, in fact, growing bacteria at elevated temperatures is a common curing method (*Summers,* 1996). It has also been described that during starvation, the bacterium may degrade its plasmids as a source of energy and nucleic acids (*Caldwell et al.* 1989). In general, the *V. anguillarum* plas-

Table 2. Loss of plasmids and viability of *Vibrio anguillarum* stored at different temperatures[1].

Strain	Temp.	Time of storage, months 1	2	3	6	9	18
VIB 1	5 °C						ng[2]
	20 °C						ng
	37 °C	ng	wg[3]	ng	ng	ng	ng
VIB 18	5 °C						ng
	20 °C				wg		ng
	37 °C	wg	wg	ng	ng	ng	ng
VIB 56	5 °C				ng	ng	ng
	20 °C						
	37 °C	ng	wg	ng	ng	ng	ng
VIB 103	5 °C	200 kb lost	ng	ng	ng	ng	ng
	20 °C			ng	ng	ng	ng
	37 °C	ng	ng	ng	ng	ng	ng
VIB 188	5 °C			ng	ng	ng	ng
	20 °C			wg	ng	ng	ng
	37 °C	ng	ng	ng	ng	ng	ng
VIB 207	5 °C				ng	ng	ng
	20 °C				ng	ng	ng
	37 °C	wg	ng	ng	ng	ng	ng
VIB 225	5 °C				ng	ng	ng
	20 °C			ng	ng	ng	ng
	37 °C	ng	ng	ng	ng	ng	ng
VIB 243	5 °C				ng	ng	ng
	20 °C				wg		
	37 °C	67 kb lost	ng	ng	ng	ng	ng

[1] empty cells = no plasmids lost.
[2] ng = no growth.
[3] wg = weak growth.

mids were stable at storage. Only in 2 cases were a plasmid lost, one at 5 °C and one at 37 °C. It should be mentioned, though, that the method used had some statistical limitations. Usually, only a single colony was tested for plasmid content, and thus, a low percentage of plasmid loss could have gone undetected. A statistically better result would have been obtained by always screening several colonies from each strain. In this experiment this was only done on a single occation, where 10 colonies from each strain were tested, and these 10 colonies of each strain all had identical plasmid profile. The cultures were only viable for a limited period at 5 °C, 20 °C and – in particular – 37 °C. the conclusion of this would be that stock cultures of *V. anguillarum* should always be kept at –80 °C, and care should be taken when sending strains from one laboratory to another; especially during warm summer periods, such shipping may affect both viability and plasmid content. After 18 months of storage, only 2 cultures kept at 20 °C were viable, but none at 5 °C or 37 °C, indicating that room temperature is the best temperature to keep working cultures. *Olsen et al.* (1994) reported no problems with viability of the *Salmonella* strains included in their experiment.

Subculturing experiments

Most plasmids were stable during all 100 subculturings. Only 2 cultures lost a plasmid after 73 and 31 subculturings, respectively. This indicates that *V. anguillarum* plasmids are in general stable during standard laboratory handling, which also comprizes subculturing, but that plasmids may be lost on a few occasions during such procedures.

Mixed cultures

The presence or absence of the pJM1-like plasmid did not seem to affect growth rate in vitro. In mixed cultures of a plasmid-carrying and a plasmid-free strain, both strains were recovered in almost equal numbers. This is in contrast to the results of studies of a number of other bacteria. Thus, in a study of *Vibrio harveyi,* (*Hoyt & Sizemore* 1982) a plasmid-carrying, bacteriocin-producing strain dominated over a cured mutant in a mixed culture, indicating that the plasmid-encoded bacteriocin was important in the competition against other strains. In contrast, in a study of a plasmid-carrying Tet^r strain of *Lactobacillus reuteri,* (*Sarra et al.* 1989) the cured, tetracyclin sensitive strain dominated over the parent strain in competition experiments using mixed cultures, and in pure cultures, the cured strain grew faster and reached higher end cell density than the parent strain. These results show that plasmids can be beneficial for the growth of the bacterium that carries them or they can be a burden that inhibit growth. However, neither seemed to be the case for the pJM1-like plasmid under non-selective conditions in vitro. The χ^2 results showed no differences in the numbers of plasmid-containing and plasmid-free cells in the mixed cultures. This also means that the growth rates of the plasmid containing and plasmid free cultures are the same.

The present investigation indicates that plasmids of *V. anguillarum* are relatively stable *in vitro.* However, their stability in vivo has not been studied. As mentioned earlier, plasmid instability or rather plasmid variability has been observed in other fish pathogens, *V. damsela* (*Pedersen et al.* 1997a) and atypical *A. salmonicida* (*Pedersen et al.* 1996a). Likewise, the resistance of *V. anguillarum* plasmids to various curing methods needs to be further investigated.

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Sammendrag

Overlevelse under opbevaring samt stabilitet af plasmider under opbevaring og subkultivering for stammer af Vibrio anguillarum.

Stabiliteten af plasmider, 3,3 – ~200 kb, hos 8 *Vibrio anguillarum* stammer med forskellige plasmidprofiler og reaktioner med O-antisera blev studeret over en 18 måneders periode. Stammer, der blev opbevaret ved 37 °C, holdt sig imidlertid kun levende i en kort periode på højst 2 måneder, mens stammer opbevaret ved 5 °C og 20 °C holdt sig levende i længere tid. Levende stammer bibeholdt til gengæld deres plasmidprofiler gennem hele eksperimentet, bortset fra 2 tilfælde, hvor henholdsvis et 67 kb og et 200 kb plasmid blev mistet. Stammer opbevaret ved –80 °C holdt sig alle levende og opretholdt deres plasmidprofil gennem hele studiet. Ved subkultivering dagligt i op til 100 på hinanden følgende dage beholdt 6 ud af 8 stammer deres plasmidprofil. Kun 2 stammer mistede et plasmid. Ved opsamling af 100 enkeltliggende kolonier fra agar plader havde alle kolonier samme plasmidindhold. Resultaterne tyder på, at plasmidprofiler hos *V. anguillarium* er meget stabile overfor subkultivering, opbevaring og laboratoriemæssig håndtering ved anvendelse af standard laboratorie procedurer, og derfor pålidelige som markører ved epidemiologiske undersøgelser. I et andet eksperiment blev 2 par af stammer dyrket sammen i blandingskulturer. De var helt identiske, bortset fra, at den ene stamme i hvert par indeholdt det 67 kb pJM1-lignende virulens plasmid, mens den anden havde mistet dette plasmid. Resultaterne heraf viste,

at vækstraten var ens for stammer med og uden plasmid. Dette tyder på, at i hvert fald under laboratorieforhold er dette plasmid hverken til fordel eller ulempe for bakteriens vækst.

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