Expression of foreign genes and selection of promoter sequences in *Acholeplasma laidlawii*

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The stable maintenance and expression of foreign genes in mollicutes (mycoplasmas) have been difficult to achieve due to the lack of suitable vectors. In this paper we show for the first time that a replicating vector can be used to express foreign genes other than antibiotic resistance genes in *Acholeplasma laidlawii*. Plasmids derived from the lactococcal vector pNZ18 could introduce and maintain four different genes for many generations in *A. laidlawii*. One of these, encoding the dominant membrane lipoprotein spiralin from the mollicute *Spiroplasma citri*, was expressed; however, expression was weak, the signal peptide of spiralin was not cleaved and the protein was not covalently modified by fatty acids. This resulted in a hydrophilic character of spiralin and its cytoplasmic localization in *A. laidlawii*. To increase the expression of foreign genes, random *A. laidlawii* DNA fragments were cloned into a pNZ18-related plasmid and expression signals were selected using the *Bacillus licheniformis* α -amylase gene as a probe. Selection was done in *Escherichia coli* as well as directly in *A. laidlawii*. Active recombinants from *E. coli* were also able to express α -amylase activities and an enzyme of native size in *A. laidlawii*. The highest activity was obtained from a recombinant selected directly in *A. laidlawii*. This is the first example of a promoter sequence selected in a mollicute. Analysis of the putative promoters in seven clones revealed similar –10 and –35 regions, and similar spacer distances in *A. laidlawii*, *Acholeplasma oculi*, *Lactococcus* and *E. coli*. Vectors related to pNZ18 should be useful for the genetic analysis of specific *A. laidlawii* proteins and functions.

Keywords: mollicutes, *Acholeplasma laidlawii*, plasmid transformation, expression, promoters

INTRODUCTION

Mollicutes (mycoplasmas) are small cell wall-less bacteria of Gram-positive origin, usually found as cell-surface parasites in humans, animals, plants and insects (Maniloff *et al.*, 1992). A marked decrease in genome size, during the evolution, has been accompanied by a change of the normal stop codon UGA to a Trp codon in several genera. The species *Acholeplasma laidlawii*, having a normal Trp codon (Tanaka *et al.*, 1989), has contributed important knowledge about basic physical properties of lipid bilayers in biological membranes (Singer &

Nicolson, 1972). We have shown that certain packing properties are metabolically maintained constant among the membrane lipids (reviewed in Rilfors *et al.*, 1993). Similar principles seem to operate in several other bacteria and organelles (Rietveld *et al.*, 1993; Wieslander *et al.*, 1994). Other special features of *A. laidlawii*, and also many other mollicutes, include modification of many membrane proteins with specific fatty acid chains (Wieslander *et al.*, 1992).

A genetic analysis of these, and other mechanisms in *A. laidlawii*, demands efficient vectors and a good expression of introduced genes other than the genes involved in vector functions. Neither have been obtained (Dybvig, 1992). Hence, development of plasmid and other vectors adapted to *A. laidlawii* are needed. A lactococcal plasmid (pNZ18), able to replicate in several Gram-positive bacteria and in *Escherichia coli*, yielded acceptable trans-

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formation frequencies and was stably maintained in *A. laidlawii* (Sundström & Wieslander, 1990). This report deals with the cloning of foreign genes from which full-size proteins were expressed in *A. laidlawii*, as well as the selection of suitable promoter sequences, using plasmids related to pNZ18. The following genes coding for different types of proteins were tested for expression in *A. laidlawii*: the spiralin gene encoding the major membrane lipoprotein in *Spiroplasma citri* (Wróblewski *et al.*, 1977; Mouchès *et al.*, 1985; Bové *et al.*, 1993); an *A. laidlawii* gene coding for E2p, a subunit of the pyruvate dehydrogenase enzyme complex (Wallbrandt *et al.*, 1992); a β -lactamase gene from *E. coli*; and an α -amylase gene from *Bacillus licheniformis*. The two latter genes are efficient and much used probes for membrane protein topology (Broome-Smith *et al.*, 1990) and secretion studies (Simonen & Palva, 1993), respectively. *A. laidlawii* promoter sequences were selected in both *A. laidlawii* and *E. coli* with the help of a promoter-less α -amylase gene. These selected sequences will be useful in increasing the expression of heterologous genes in *A. laidlawii*.

METHODS

Bacterial strains and culture conditions. *A. laidlawii* strain 8195 (Sladek *et al.*, 1986), clone 2501, which can be transformed to a relatively high frequency (Sundström & Wieslander, 1990), was used. The cells were grown without shaking at 37 °C for 24–48 h (2–10%, v/v, inoculation), in a BSA-tryptose medium (Wallbrandt *et al.*, 1992) supplemented with 75 μ M palmitic acid and 75 μ M oleic acid. Solid medium was made by addition of 1.2% (w/v) agar. *E. coli* MC1061 (Casadaban & Cohen, 1980) was grown with shaking at 37 °C overnight in Luria-Bertani medium. *S. citri* Maroc R8A2^T (ATCC 27556^T; Saglio *et al.*, 1973) was grown without shaking at 32 °C for 48 h (10% inoculation) in BSR medium (Whitcomb, 1983).

Labelling with radioactive fatty acids. *A. laidlawii* medium was supplemented with 30 mCi l⁻¹ (1.11 GBq l⁻¹) (1.36 μ M) ³H-labelled or 2 mCi l⁻¹ (74 MBq l⁻¹) (37.0 μ M) ¹⁴C-labelled palmitic acid (New England Nuclear). PMSF was added to 1 mM in all solutions in the harvesting procedure (below). Membrane lipids were extracted with 20 volumes ice-cold ethanol overnight at -20 °C. The proteins were sedimented by centrifugation. The extraction was continued with cold ethanol followed by ethanol/diethyl ether (1:1, v/v) and diethyl ether. Proteins in pellets were analysed by SDS-PAGE (see below) and immunoblotting. Gels and immunoblot membranes were processed for fluorography or autoradiography by standard procedures.

Preparation of *A. laidlawii* membrane and cytoplasmic fractions. *A. laidlawii* cells were harvested by centrifugation, washed with β -buffer (0.15 M NaCl, 0.05 M Tris/HCl, pH 7.4), frozen in water overnight, and lysed by stirring for 2 h at 37 °C as in Nyström *et al.* (1986). Membranes were collected by centrifugation at 48000 g_{max} for 1.5 h at 5 °C and washed once in diluted β -buffer (1:20, v/v). The supernatant (cytoplasm) was lyophilized. For some studies of spiralin, the cytoplasmic fraction was separated from small amounts of contaminating membrane proteins by ultracentrifugation twice at 265000 g_{max} for 30 min at 4 °C. For α -amylase analysis, cells were grown for 24 h, washed and suspended in β -buffer, lysed by sonication (2 $\times$ 1 min at 70 W and 0 °C), and centrifuged (12000 g_{max} for 30 min at 4 °C) to separate fractions enriched in membrane and cytoplasm.

Plasmids. The plasmids pNZ18 and pNZ19 (Table 1 and Fig. 1a) are modified *Lactococcus lactis* plasmids with a broad host-range (de Vos, 1987; Sundström & Wieslander, 1990). Both plasmids are derived from pNZ12. The plasmid pNZ18H was constructed by inserting a *Hind*III linker with the sequence 5'-CAAGCTTG-3' in the *Pvu*II site in pNZ18.

Plasmid pGIP312 (Table 1 and Fig. 1b) (Hols *et al.*, 1992) contains a truncated α -amylase gene (*amyL*) from *B. licheniformis* (Piggot *et al.*, 1984). The α -amylase gene lacks a promoter, RBS and the 5'-fragment encoding the signal peptide. The replicon and two of the resistance genes are from pNZ12. A terminator from the phage T4 gene 32 is inserted in front of the α -amylase gene (Prentki & Krisch, 1984).

Cloning of genes. A DNA fragment (968 bp) containing the spiralin gene and its proposed promoter, RBS and transcription terminator was amplified from plasmid pES1 (Mouchès *et al.*, 1985; Chevalier *et al.*, 1990a) by PCR. The primer for the 3' end was modified with a *Hind*III site and the other primer with a *Pst*I site. Plasmid pNZ18H and the PCR products were cut with *Hind*III and *Pst*I and ligated together, yielding plasmid pNZ18HS. A larger *Sac*I/*Bst*EII fragment was cut out of pNZ18HS, and ligated into pNZ19 (Fig. 1a).

Random fragments of *A. laidlawii* genomic DNA partially digested with *Sau*3A, *Alu*I or *Rsa*I, have been inserted in front of a truncated β -lactamase gene (T. K. Jarhede *et al.*, unpublished results) in the signal sequence selection vector pGPB14 (Smith *et al.*, 1987). From one of the selected clones (pALE102), the *A. laidlawii* insert (1.3 kbp) and the in-frame β -lactamase gene (1.3 kbp) was excised and ligated into pNZ18H.

A 3'-truncated dihydrolipoamide acetyltransferase (E2p) gene from *A. laidlawii* (Wallbrandt *et al.*, 1992) was excised from the plasmid pB15 (Tegman *et al.*, 1987) and ligated into pNZ18H. The 3'-truncated form of the gene was used to separate the cloned E2p from the naturally occurring E2p. It encodes a peptide of 43 kDa instead of 75 kDa for the native one (as judged from SDS-PAGE).

To construct a library of expression signals from *A. laidlawii*, genomic DNA was partially digested with *Sau*3A. The fragments obtained were ligated into a unique *Bam*HI site in front of the truncated α -amylase gene of pGIP312 (Fig. 1b). Expression of the *amyL* gene was first assayed in *E. coli* by iodine plate tests (see below). The plasmids with random inserts initially selected in *E. coli* were also used to transform *A. laidlawii* cells. The transformants were analysed for the production of α -amylase in starch-agar plates (see below). In another experiment, *A. laidlawii* cells were directly transformed by plasmids with inserted *Sau*3A fragments. Plasmids from the positive clones found here were also used to transform *E. coli*.

Plasmid preparations and transformation of cells. Plasmids were isolated according to the alkaline lysis method (Sambrook *et al.*, 1989). Transformation of competent *E. coli* (Hanahan, 1985) was performed as described by Sambrook *et al.* (1989). Transformants were selected on agar plates containing 10 μ g chloramphenicol ml⁻¹ or 10 μ g chloramphenicol ml⁻¹ plus 12.5 μ g streptomycin ml⁻¹. PEG-dependent transformation of *A. laidlawii* was done as described by Sladek & Maniloff (1983) with modifications as in Sundström & Wieslander (1990). Neomycin (40 μ g ml⁻¹) was used in the agar plates. The transformation frequency was 4 $\times$ 10⁻⁴ c.f.u.⁻¹, which corresponded to 4 $\times$ 10³ transformants per μ g DNA; non-transformed cells could not grow on plates with neomycin.

Table 1. Plasmids used in this study

Plasmids	Size (kbp)	Genes and relevant features	Reference
pNZ12	4.3	Promiscuous <i>L. lactis</i> plasmid	de Vos (1987)
pNZ18	5.7	Derivate of pNZ12 with cloning cassette from pJRD158	de Vos (1987); de Vos, personal communication
pNZ18H	5.7	pNZ18 with <i>HindIII</i> site instead of <i>PvuII</i>	This paper
pNZ19	5.7	As pNZ18 but orientation of <i>XhoI/SalI</i> fragment of pJRD158 is reversed	de Vos, personal communication
pES1	11.4	pBR328 vector with spiralin gene, <i>rpsB</i> , <i>tsf</i> , <i>pfk</i> and <i>pyk</i> from <i>S. citri</i>	Chevalier <i>et al.</i> (1990a); Mouchès <i>et al.</i> (1985)
pGIP312	8.3	Expression/secretion signal vector carrying the truncated <i>amyL</i> gene and the same replicon as pNZ18	Hols <i>et al.</i> (1992)
pGPB14	5.7	Expression/secretion signal vector with truncated β -lactamase gene	Smith <i>et al.</i> (1987)
pB15	7.9	Vector pAT153 (pBR322 derivative) with pyruvate dehydrogenase operon	Tegman <i>et al.</i> (1987); Wallbrandt <i>et al.</i> (1992)
pNZ18HS	6.2	pNZ18H with spiralin gene from pES1	This paper
pNZ19HS	6.2	pNZ19 with spiralin gene from pNZ18HS	This paper
pNZ18H.102	8.3	pNZ18H with β -lactamase gene from pGPB14 and an <i>A. laidlawii</i> DNA fragment	This paper
pNZ18H.E2p	8.2	pNZ18H with a truncated E2p from pB15	This paper
pALP30	8.3	pGIP312 with inserted small <i>A. laidlawii</i> DNA fragment. Selected in <i>A. laidlawii</i>	This paper
pALP35 pALP52 pALPA pALB pALPI pALPJ	8.3	pGIP312 with inserted small <i>A. laidlawii</i> DNA fragments. Selected in <i>E. coli</i>	This paper

SDS-PAGE and immunoblotting. Samples with spiralin were separated in SDS-polyacrylamide gels in a modified Neville system (Nyström *et al.*, 1986). Expression of α -amylase was analysed in 10% (w/v) polyacrylamide gels (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue, assayed for α -amylase activity or semi-dry electroblotted onto Immobilon-P transfer membranes (Millipore) (Wallbrandt *et al.*, 1992). Primary antibodies (polyclonal and monoclonal, kindly provided by H. Wróblewski, Rennes, France) directed against *S. citri* spiralin, and alkaline phosphatase- or peroxidase-conjugated secondary antibodies were used.

Electroelution and N-terminal amino acid sequencing. Preparative gels (thickness 1.5 mm) were aged 24 h before electrophoresis. A gel slice corresponding to the spiralin band was excised from stained gels and stored frozen before

electroelution (BioRad electro-eluter model 422) in 50 mM NH_4HCO_3 and 0.1% SDS. The samples were then electro-dialysed in the same equipment in 50 mM NH_4HCO_3 and 0.01% SDS to reduce the amount of SDS. Samples from 10 gels were pooled, lyophilized and separated by another SDS-PAGE (as above). Processing of proteins for amino acid sequencing was performed essentially as described by Matsudaira (1987) and by Nyström *et al.* (1992). Proteins were semi-dry blotted onto Trans-Blot transfer membranes (BioRad) and processed as described by the manufacturer. A strip of the membrane, corresponding to the visualized spiralin band was cut out and subjected to sequencing.

Determination of the N-terminal amino acid sequence of spiralin was performed with a gas-phase sequencer (Applied Biosystem 477A) equipped with an on-line phenylthiohydantoin amino

Fig. 1. Restriction maps of plasmids. (a) pNZ19 with the spiralin gene, replicon from *L. lactis* plasmid pSH71, and chloramphenicol (*cam*) and neomycin (*neo*)/kanamycin (*kan*) resistance genes from the *Staphylococcus aureus* plasmids pUB110 and pC194 (de Vos, 1987; de Vos, personal communication). (b) pGIP312 according to Hols *et al.*, 1992. Streptomycin (*stm*) resistance gene from the *E. coli* plasmid pMTL23P (Chambers *et al.*, 1988).

acid analyser (Applied Biosystem 120A), at the Department of Medical Biochemistry and Biophysics, Umeå University, Sweden.

Triton X-114 phase separation. Triton X-114 solubilization and temperature phase separation (Bordier, 1981) of membrane fractions from *S. citri* and *A. laidlawii*, and the cytoplasmic fraction (ultracentrifuged) from *A. laidlawii*, were performed essentially as described by Nyström *et al.* (1992).

Assays of α -amylase activity. Bacteria were grown on appropriate agar plates supplemented with 0.2% soluble starch. Amylase producing colonies were detected by staining of the starch with iodine reagent (Smith *et al.*, 1987). To obtain colony replicas, cells were transferred to new plates with nitrocellulose filters (Hybond-C, Amersham) as described by Sundström & Wieslander (1990). Detection of α -amylase activity in SDS-polyacrylamide gels containing 0.25% soluble starch was done with the iodine reagent as described by Smith *et al.* (1987, 1988) after rinsing the gel four times with 20 mM potassium phosphate buffer/50 mM NaCl (pH 7.4) supplemented with 0.25% soluble starch. Amylase activity in liquid solutions was determined spectrophotometrically with Starch Azure (Sigma) as a substrate (Smith *et al.*, 1988).

DNA sequencing. The DNA sequences of one strand of the inserts in the α -amylase-producing clones were obtained several times by the dideoxy chain-termination method of Sanger *et al.* (1977) using the Sequenase version 2 DNA sequencing kit (USB). Double-stranded plasmid DNA was used as templates together with synthesized oligonucleotide primers.

RESULTS

Plasmid stability

The genes for spiralin, a hybrid β -lactamase, and truncated E2p were cloned in pNZ18H or pNZ19. These recombinant plasmids, isolated after transformation into both *E. coli* and *A. laidlawii* were of expected sizes and did not change over time as analysed by restriction enzyme cleavage and gel electrophoresis. pNZ19 with the inserted spiralin gene was structurally and segregationally stable for at least 11 successive inoculations which correspond to about 100 generations.

Expression of foreign genes

The spiralin, β -lactamase, truncated E2p and α -amylase genes could be expressed in *E. coli*. The first three proteins were detected by polyclonal antibodies, and β -lactamase and α -amylase were also detected by activity measurements. Spiralin (Fig. 2) and α -amylase (see below and Fig. 3), but not the β -lactamase nor the truncated E2p, could also be detected in *A. laidlawii*. Expression of the spiralin gene in *A. laidlawii* was observed in cells harbouring the spiralin gene in pNZ19 (called pNZ19HS) but not in cells with the spiralin gene in pNZ18H. The orientation of the spiralin gene in pNZ19HS is shown in Fig. 1a; the spiralin gene cloned in pNZ18H is oriented in the opposite direction. It was thus only expressed when the gene was oriented in the same direction as the *repA* gene of the vector. The spiralin from pNZ19HS could be detected with both polyclonal (Fig. 2) and monoclonal antibodies directed against purified *S. citri* spiralin, but the level of expression was rather low both in *A. laidlawii* and in *E. coli* compared to that in *S. citri*. This observation indicates that the transcription and/or translation signals

Fig. 2. Expression of spiralin in *A. laidlawii*. Semi-dry blot filter from an 18% SDS-polyacrylamide gel which had been run at 120 V for 24 h. The filter was incubated with polyclonal antibodies against *S. citri* spiralin. Lane 1: cytoplasm from *A. laidlawii* transformed with pNZ19HS. Arrowhead indicates spiralin. Lane 2: cytoplasm from *A. laidlawii* transformed with pNZ18 vector.

which govern spiralin expression are less efficient in *A. laidlawii* than in *S. citri*.

Localization and processing of spiralin

Spiralin from *S. citri* appeared as a double band in our gel system with apparent molecular masses of about 26.7 and 25.4 kDa. Under the same conditions the molecular masses of spiralin produced in *A. laidlawii* and *E. coli* were estimated to be 31.7 kDa and 32.6 kDa, respectively. The N-terminal amino acids of spiralin prepared from transformed *A. laidlawii* were sequenced and the order of the six amino acids obtained (Met Lys Lys Leu Leu Ser) was identical to the N-terminal part of the signal sequence in spiralin from *S. citri*, as deduced from its gene sequence (Chevalier *et al.*, 1990a; Le Hénaff *et al.*, 1991). Hence, the signal peptide seemed to be retained and this could explain the larger size of the protein.

The spiralin in *A. laidlawii* was found predominantly in the cytoplasmic fraction, even if this fraction was purified from contaminating membranes by two extra ultracentrifugation steps. Only a small proportion was detected in the membrane by immunoblotting. This may result from inefficient targeting due to the absence of acyl chain modification. *A. laidlawii*, with or without the spiralin gene, was grown in the presence of highly labelled [^3H] or [^{14}C]palmitic acid (16:0), but no labelling of spiralin could be detected (data not shown).

The hydrophobic properties of spiralin from *A. laidlawii* containing the signal peptide but lacking lipid modification, was analysed by Triton X-114 phase partitioning. Spiralin from *S. citri* and *Spiroplasma melliferum* partitioned in the detergent (bottom) phase in the Triton

Fig. 3. α -Amylase activity in an SDS-polyacrylamide gel. The samples are cell lysates of *A. laidlawii* transformed with the following plasmids: Lanes: 2, pALP-52; 3, pALP-30; 4, pALP-21; 5, pALP-35. Lane 1, untransformed *A. laidlawii* cell lysate as a negative control.

X-114 phase separation as would be expected for lipid-modified membrane proteins. On the other hand spiralin from *A. laidlawii* cytoplasm remained in the aqueous phase. A major *A. laidlawii* membrane acyl protein (T_2) was used as a control. This protein partitioned in the detergent bottom phase as expected and observed before (Nyström *et al.*, 1992).

Selection of promoter sequences

The expression of spiralin in *A. laidlawii* was low and two other proteins (β -lactamase, truncated E2p) were not expressed to detectable levels. Are the expression and translation signals for the genes used much different from the *A. laidlawii* ones? It has been shown that there are considerable differences in the efficiency of certain promoters when used with different lactic bacteria, indicating that endogenous promoters should be used when optimising expression (de Vos & Simons, 1994). To select *A. laidlawii* endogenous signals, random *A. laidlawii* DNA fragments were cloned in pGIP312. An inserted DNA fragment which contains a promoter, RBS and start codon for translation, and is cloned in the proper *amyL* ORF of pGIP312, directs the synthesis of α -amylase. When this enzyme is synthesized, colonies and the surrounding halo areas become clear if cells are grown on starch-agar plates and later stained with an iodine reagent. The level and localization of α -amylase would depend on (i) the strength of the promoter sequence and (ii) the potential signal peptide encoded by the inserted DNA fragment (cf. Hols *et al.*, 1992). When selection was done in *E. coli*, 0.6% of the observed colonies presented a starch-debranching activity. Three of these plasmids (pALP-21, pALP-35 and pALP-52) were used to transform *A. laidlawii*. Cells containing any of these three recombinant plasmids expressed the *amyL* gene, yielding active α -amylase (Table 2, Fig. 3). The amount of α -amylase was in all cases much lower than in *E. coli*, but so were the growth rate, cell size and colony size.

In another experiment, *A. laidlawii* cells (clone 2501) were directly transformed by pGIP312, having inserted *Sau3A*

Table 2. α -Amylase activity in cell lysate of transformed *A. laidlawii*

Plasmid	Activity*
pGIP312	1.01 $\pm$ 0.32
pALP-21	8.36 $\pm$ 0.30
pALP-30	11.11 $\pm$ 0.30
pALP-35	7.18 $\pm$ 0.35
pALP-52	9.76 $\pm$ 0.46

* Activity is expressed as α -amylase units per OD₅₄₀ unit of cells. One unit increases OD₅₉₅ by one unit h⁻¹ by enzymic hydrolysis of starch azure. Activity was measured in three experiments.

fragments from the genome of *A. laidlawii*. Previously it had been necessary to pass recombinant plasmids through *E. coli* to amplify the plasmid DNA before transformation of mollicutes, because much plasmid DNA is needed. *A. laidlawii* clone 2501 can be transformed with, for mollicutes, a relatively high frequency. The direct transformation of *A. laidlawii* by recombinant plasmids gave one colony that produced α -amylase, pALP-30. The insert of pALP-30 gave stronger activity in *A. laidlawii* than the

ones selected in *E. coli* (Table 2). *E. coli* transformed with pALP-30 also produced α -amylase.

Cells were fractionated to estimate the α -amylase content in the growth medium, and in the membrane and cytoplasmic fractions. These fractions were analysed for activity of α -amylase in SDS-polyacrylamide gels. The apparent molecular mass was 50–55 kDa, close to that in *B. licheniformis*. No starch-debranching activity was found in the growth medium supernatant or in the isolated membrane fraction. The α -amylase was only detected in the cell lysate (Fig. 3). Thus, the translation product seemed to be retained in the cytoplasm, and the α -amylase was not exported.

A. laidlawii sequences

The production of active α -amylase in both *E. coli* and in *A. laidlawii* indicated that expression signals had been cloned that were well recognized by the transcription/translation machinery in both kind of cells. Seven of the DNA inserts were sequenced and ORFs in the same frame as α -amylase and start codons with properly spaced RBS were found. On average, 6.4 bases in the putative RBS (Table 3) could form basepairs with the 3' end of the 16S rRNA of *A. laidlawii* (Woese *et al.*, 1980). In *E. coli* and *B. subtilis* the corresponding values are 3–4 and 8.7 bases,

Table 3. Putative promoters selected from *A. laidlawii*

Plasmid*	–35 Region†‡	Distance in bases	–10 Region‡	RBS§
pALP-30	TTGAat	12	TATAtT	<u>ATCCAGGACAAACT</u>
	TTGAat	17	TtcAcT	<u>ATCCAGGACAAACT</u>
pALP-52	TTGAtg	17	TgTtAc	<u>ATGAGGGAAGCGGT</u>
pALP-21	TTGAgt	18	TATAcg	<u>ACGGATG</u>
pALP-A	TTGgtA	17	TAAaAc	<u>AGGAAGGTGGT</u>
pALP-B	TaGAaA	18	TATtAT	<u>AGAACTGGTAA</u>
	TaGACA	15	TAAAgT	<u>AGAACTGGTAA</u>
pALP-I	TTGtag	17	TATAtT	<u>ATGGGTG</u>
pALP-J	TTtACT	18	TATAcc	<u>ATTAGGGGAGA</u>
	TaatCA	18	TATAAT	<u>ATTAGGGGAGA</u>
<i>S. citri</i> spiralin	TTatTT	20	TgTAAT	<u>AGAAAGGAAAT</u>
<i>A. laidlawii</i> consensus¶	TTG AnA	Mean 17	TATA AT	<u>AGAAAGGAGGTGAT</u>
<i>A. oculi</i> consensus¶**	TTcA tn	17.5	TATA AW	
<i>E. coli</i> consensus¶	TTG ACA	17	TATA AT	<u>TAAGGAGGTGATC</u>
Lactococcal consensus††	TTG AnA	17.5	TATA AT	

* Plasmids pALP-A, -B, -I and -J were selected and tested in *E. coli* only.

† The –35 sequences were identified visually and by the PC/GENE program SIGNAL in all *A. laidlawii* sequences except in pALP-A and the second -J.

‡ Bases identical to the *E. coli* –35 or –10 consensus regions are shown in capital letters.

§ Bases that are complementary to the 3' end of *A. laidlawii* 16S RNA (Woese *et al.*, 1980) are underlined.

|| Chevalier *et al.* (1990a).

¶ Bold letters in the consensus indicates that that base was found in that position in at least 70 % of the sequences and normal letters indicate that it was found in at least 40 %. W = A or T; n = any base.

** Knudtson & Minion (1994).

†† Sequences from de Vos & Simons (1994).

respectively. The distance between the RBS and the start codon (calculated as in Maniloff *et al.*, 1994) was between 8 and 14 bp, except in pALP-A which had 18 bp but this clone gave a low α -amylase activity in *E. coli*. In the mycoplasma virus L2 the distance was 6–15 bp (Maniloff *et al.*, 1994). Since all the selected promoter regions also functioned in *E. coli*, an obvious way to analyse them was to look for promoter sequences with similarities to the *E. coli* consensus –35 TTGACA and –10 TATAAT sequences. The –35 sequences were identified with the PC/GENE program SIGNAL and visually. The –10 regions were then searched for at a plausible distance from the –35 regions. Putative promoter sequences were found in all inserts (Table 3). For sequences pALP-B and -J two tandem promoters were identified (Table 3). One of the –10 regions given for pALP-30 may not be functional due to there being a very short distance (only 12 bases) to the –35 region. Our other sequences had spacer regions with 15–18 bases; in *E. coli* the distance is 15–21. A consensus promoter, in which the bases at each position were those most frequently found in the *A. laidlawii* –10 and –35 regions, was derived (Table 3).

Most of the translated protein sequences found in-frame with the α -amylase did not have the features of typical signal peptides. This was corroborated by the absence of α -amylase activity in the growth medium and membrane fraction of transformed *A. laidlawii* cells. The α -amylase was, however, active without a signal peptide.

DISCUSSION

This study shows that pNZ19 and pGIP312 could be used as expression and selection vectors in *A. laidlawii*. We have cloned and expressed a gene encoding the foreign protein spiralin in *A. laidlawii*. We have also selected *A. laidlawii* DNA fragments containing potential promoters, Shine-Dalgarno sequences and start codons. Stability of vectors and cloned genes have previously been a problem in mollicutes. The streptococcal plasmids pVA868 and pVA920 formed deletion derivatives in *A. laidlawii* (Dybvig, 1989), and SpV1, with the G-fragment of the cytoadhesin P1 protein from *Mycoplasma pneumoniae* lost the cloned insert (Marais *et al.*, 1993). The plasmids pNZ18H, pNZ19 and pGIP312 with inserted DNA were all segregationally and structurally stable. Since pNZ19 and pGIP312 are broad-host-range plasmids and have an origin of replication and a *repA* gene that have high sequence similarities to the corresponding regions of a mycoplasma plasmid pADB201 (de Vos *et al.*, 1989), it is likely that they may function as vectors in other mollicutes as well, provided that there are no interfering UGA codons in the vector.

Spiralin in *A. laidlawii*

In *S. melliferum*, spiralin is modified at the N-terminal cysteine with two ester-linked 16 carbon chains and one amide-linked 14-carbon chain, and the signal peptide is cleaved off by an enzyme analogous to the *E. coli* signal peptidase II (Wróblewski *et al.*, 1989; Le Hénaff, 1992; M. Le Hénaff & H. Wróblewski, unpublished results). The

spiralins of *S. citri* and *S. melliferum* are 75% identical in the amino acid sequences, and the sequences around the cleavage sites are identical (Chevalier *et al.*, 1990b). It is therefore anticipated that the spiralin from *S. citri* is modified and cleaved in the same way as the *S. melliferum* spiralin. The larger size of spiralin in *A. laidlawii* is probably due to an uncleaved signal peptide. Retained signal peptides have also been observed for other bacterial lipoproteins when cloned in foreign hosts, e.g. the signal peptide of the mycoplasma lipoprotein VlpC was not cleaved in *E. coli* (Cleavinger *et al.*, 1994). Our results also indicate that spiralin in *A. laidlawii* is not fatty acylated as in the native host. In *S. melliferum* the spiralin is anchored to the outer surface of the membrane with acyl tails (Wróblewski *et al.*, 1989; Le Hénaff, 1992; M. Le Hénaff & H. Wróblewski, unpublished results). The spiralin in *A. laidlawii* was mainly found in the cytoplasm. Thus, it seems that the signal peptide is not enough for membrane anchorage and acyl chains are also needed. These observations also indicate that the processing signals of spiralin were not recognized by the *A. laidlawii* cells.

Spiralin constitutes 22% (w/w) of the protein in the membrane of *S. citri* (Wróblewski *et al.*, 1977). However in *A. laidlawii* and in *E. coli* the amounts were much lower. Over-expression of membrane proteins in *E. coli* has proven to be more difficult than overproduction of soluble proteins (Schertler, 1992). Since spiralin is not processed as in spiroplasmas (see above), this could limit membrane insertion which in turn could prevent expression. The expression of other cloned proteins in mollicutes has also been low. The G-fragment of the cytoadhesin P1 protein could be detected with antibodies but not visualized in a Coomassie-stained gel when expressed in *S. citri* (Marais *et al.*, 1993). Activity of the kanamycin/neomycin nucleotidyl transferase (encoded by the resistance gene in the vector) could be detected in *A. laidlawii* but the amounts of this protein were also too low to be visualized by Coomassie staining. The low expression in *A. laidlawii* could be due to possible differences in promoter and upstream element usage. Very few promoters have so far been sequenced from *A. laidlawii* genes.

A. laidlawii promoter sequences

The selection of functional *A. laidlawii* promoter, RBS and start codon was achieved with a truncated α -amylase gene. This has been used previously as a reporter gene to find expression and secretion signals from *Enterococcus faecalis* (Hols *et al.*, 1992), *B. subtilis* (Smith *et al.*, 1987; 1988) and *L. lactis* DNA (Perez-Martinez *et al.*, 1992). Selection in either *E. coli* or *A. laidlawii* gave expression of α -amylase in re-transformed *A. laidlawii* and *E. coli* for all sequences that were tested. Sequences similar to *E. coli* promoter regions were found (Table 3). On average, 8.2 of the 12 bases in the proposed promoter regions were identical to the *E. coli* consensus, a value similar to that found for *E. coli* promoters. There was a higher similarity to the *E. coli* –10 than to the –35 region; this has been observed in other mollicute promoter regions (Muto *et al.*, 1992), and could be due to the high mol% of adenine

and thymine in the genome of these bacteria. The *A. laidlawii* putative promoter sequences are also very similar to the promoters selected from *A. oculi* (Knudtson & Minion, 1994) and *Lactococcus* sequences (de Vos *et al.*, 1994; Table 3). However, in contrast to our study only 10% of the *A. oculi* sequences selected in *E. coli* were functional in *A. oculi* (Knudtson & Minion, 1994). It is noteworthy that the sequence selected directly in *A. laidlawii* gave the highest α -amylase activity in this organism (Table 2). The promoter of spiralin (Table 3) has a longer spacer, and the -35 region a lower similarity, to the *E. coli* promoter than the selected putative *A. laidlawii* promoters. This could be one reason for the low expression of spiralin in *A. laidlawii* and *E. coli*, another reason could be reduced stability of the mRNA. Since the sequences that are functional in *A. laidlawii* are so similar to the *E. coli* consensus promoters, this can not explain the difficulties in expressing *E. coli* genes in mollicutes. It has been suggested that regions upstream of the promoter contribute to the efficiency of transcription initiation in *L. lactis* (de Vos & Simons, 1994). We do not know if any other part beside the proposed -35 and -10 regions of the inserts influenced expression in *A. laidlawii*. However, we now have sequences that can promote the expression of foreign genes in *A. laidlawii* and they have been useful for the expression of an active lipid-synthesizing enzyme from *E. coli* in *A. laidlawii* (T. K. Jarhede *et al.*, unpublished results).

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