RESEARCH LETTER

Natural competence in strains of *Actinobacillus pleuropneumoniae*

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Abstract

We have identified a highly transformable strain of *Actinobacillus pleuropneumoniae* whose competence is regulated by the competence-activator Sxy as in other *Pasteurellaceae*. Other strains were poorly transformable or nontransformable. The genomes of two poorly transformable strains contain intact sets of competence genes. Moreover, we show that the low competence of one of these strains is not due to an inability to induce *sxy* expression or to a defect in Sxy function, suggesting that some other component of the competence system is defective. Although the *A. pleuropneumoniae sxy* gene has only 24% identity to its *Haemophilus influenzae* homologue, both genes fully complemented an *H. influenzae sxy* knockout, demonstrating that Sxy function is conserved throughout the *Pasteurellaceae*.

Introduction

Natural competence, the ability of many bacteria to take up DNA from their environment, is usually tightly regulated (Solomon & Grossman, 1996). Once inside the cell, the DNA can serve as a source of nutrients or can recombine with the host chromosome to transform the recipient cell. Transformation is a valuable genetic tool, but its use in the swine pathogen *Actinobacillus pleuropneumoniae* has been limited both by the poor transformability of clinically relevant strains and by our lack of understanding of competence regulation in this organism. Two strains have been reported to be naturally competent, but transformation and DNA uptake occurred at very low frequencies and competence was not detectably induced by the standard protocol used for other *Pasteurellaceae* (Rowji *et al.*, 1989; Gromkova *et al.*, 1998; Bossé *et al.*, 2004; Fujise *et al.*, 2004; Redfield *et al.*, 2006; Maughan & Redfield, 2009).

Competence regulation in Gram-negative bacteria has been best studied in the pasteurellacean *Haemophilus influenzae*, which takes up DNA under conditions of nutrient limitation (MacFadyen *et al.*, 1996, 2001; Maughan *et al.*, 2008). Genes required for DNA uptake, transport and processing form a competence regulon that is upregulated strongly when cells are transferred to the starvation medium MIV, and to a lesser extent at the onset of the stationary phase in a rich medium (Herriott *et al.*, 1970; Redfield *et al.*, 2005). These genes share a common promoter motif (CRP-S site) that requires both cyclic AMP receptor protein (CRP), the global regulator of carbon and energy metabolism, and Sxy, a specific positive regulator of competence (Zulty & Barcak, 1995; Redfield *et al.*, 2005; Cameron & Redfield, 2006).

The failure of *A. pleuropneumoniae* cultures to respond to the standard competence induction protocol is surprising in the light of recent evidence that a common mechanism of competence induction and regulation is shared not only by other *Pasteurellaceae* (*Aggregatibacter actinomycetemcomitans* and *Haemophilus parainfluenzae*) but also by species in other genera (*Vibrio cholerae* and *Escherichia coli*) (Gromkova *et al.*, 1998; Wang *et al.*, 2002; Meibom *et al.*, 2005; Cameron & Redfield, 2006; Bhattacharjee *et al.*, 2007; Sinha *et al.*, 2009). As has been observed in *H. influenzae*, expression of plasmid-borne *sxy* in these species is known to induce the CRP-S regulon and/or to cause near-constitutive natural competence. Phylogenetic analysis has shown that *A. pleuropneumoniae* and *H. influenzae* belong to two divergent pasteurellacean subclades (Redfield *et al.*, 2006), raising the possibility that the two clades regulate competence

differently. Sequenced pasteurellacean genomes in both subclades have predicted CRP-S sites preceding their competence gene homologues (Cameron & Redfield, 2006), but the regulation of Sxy and its role in their induction has not been investigated in the *A. pleuropneumoniae* subclade. Alternatively, the failure to detect competence induction may simply be an artefact of a lack of sensitivity, as the maximum transformation frequencies in the two tested strains were close to the limit of detection (Bossé *et al.*, 2004).

Competence is not typically a species-specific property: in many species, different strains are known to differ dramatically in transformability (Rowji *et al.*, 1989; Gromkova *et al.*, 1998; Fujise *et al.*, 2004; Bhattacharjee *et al.*, 2007; Maughan & Redfield, 2009). We therefore surveyed *A. pleuropneumoniae* reference strains for their ability to transform naturally. This work identified one strain whose transformation frequency was substantially higher than the previously tested strains, and whose competence is regulated similarly to that of *H. influenzae* and other *Pasteurellaceae*. To begin addressing the cause of the poor transformability of other *A. pleuropneumoniae* strains, we investigated the role of differences in Sxy expression and function.

Materials and methods

Bacterial strains and growth conditions

Actinobacillus pleuropneumoniae reference strains (listed in Table 1) and 4074 *sodC*::kan are described elsewhere (Fodor *et al.*, 1989; MacDonald & Rycroft, 1992; Nielsen *et al.*, 1997; Sheehan *et al.*, 2000; Blackall *et al.*, 2002). All strains were grown on brain–heart infusion (BHI) agar supplemented with 10% Levinthal's base (prepared as described in Bossé *et al.*, 2004) (BHI-L), or in BHI broth supplemented with 100 μg mL^{-1} β-NAD (BHI-N). *Haemophilus influenzae* strains Rd KW20, *sxy*::kan and MAP7 (Zulty & Barcak, 1995; Poje & Redfield, 2003) were grown in BHI broth or agar supplemented with 10 μg mL^{-1} hemin and 2 μg mL^{-1} NAD (BHI-HN). All strains were grown at 37 °C. Where necessary, the media were supplemented with 50 μg mL^{-1} kanamycin, 1 μg mL^{-1} chloramphenicol or 2.5 μg mL^{-1} novobiocin.

Table 1. Transformability of *Actinobacillus pleuropneumoniae* strains

Strain name	Serotype	Transformability	Transformation frequency ± SD*
Shope 4074	1	Low	$4.3^{-08} \pm 2.0^{-08}$
1536	2	None detected	$< 2^{-10\dagger}$
1421	3	Low	$3.6^{-08} \pm 2.7^{-08}$
M62	4	Low	$1.0^{-08} \pm 4.4^{-09}$
K17	5a	None detected	$< 2^{-09\dagger}$
L20	5b	Low	$1.6^{-08} \pm 1.1^{-09}$
Femø	6	None detected	$< 9^{-10\dagger}$
WF83	7	None detected	$< 2^{-10\dagger}$
405	8	Low	$5.8^{-08} \pm 2.1^{-08}$
CVJ13261	9	None detected	$< 2^{-10\dagger}$
D13039	10	None detected	$< 8^{-10\dagger}$
56153	11	None detected	$< 3^{-10\dagger}$
8329	12	None detected	$< 9^{-10\dagger}$
N-273	13	None detected	$< 9^{-10\dagger}$
3906	14	None detected	$< 3^{-10\dagger}$
HS143	15	High	$4.6^{-04} \pm 3.8^{-04}$
HS143*sxy*-	15	None detected	$< 3^{-10\dagger}$

*Values shown represent the mean of four assays. Transformation frequencies are the ratio of CFU mL^{-1} on BHI Kan agar to CFU mL^{-1} on BHI agar.

†Limit of detection.

Inactivation of *A. pleuropneumoniae sxy*

In order to inactivate *A. pleuropneumoniae sxy*, two regions of DNA flanking and including *A. pleuropneumoniae sxy* were PCR amplified from the chromosome of strain HS143 using primers sxyUp_F (CGGATACGGATTCTTTGGTTCTGC) and sxyUp_RBamHI (CATAGAGGGCGAACATAGGATCCTC; BamHI site underlined), and primers sxyDown_FBamHI (CTCATTGGATCCTCTCTGCTATCGAAG; BamHI site underlined) and sxyDown_R (CTACCGCAGGGATAGTTTGTT CAACC). Both PCR products were digested with BamHI, ligated using the Rapid DNA ligation kit (Roche) and reamplified using primers sxyUp_F/sxyDown_R. The resulting 1.5-kb fragment [containing a truncated *sxy* gene (194 bp deleted) with a unique BamHI site] was cloned into pGEMT (Promega) to yield plasmid psxy. In parallel, the *cat* gene from *Staphylococcus aureus* (Jansen *et al.*, 1995) was PCR amplified with primers Cm_F (CGCGGATCCGAGCTCTA**AC AAGCGGT**AAGCAGACAAGTAAGCCTCC) and Cm_R (CG CGGATCCCATGCATGCATA**ACAAGCGGT**TTCAACTAACG GGGCAGG) to incorporate the uptake signal sequence (bold) from *A. pleuropneumoniae* and BamHI restriction sites (underlined). Plasmid psxy was linearized with BamHI and the *cat* gene was inserted. The resulting plasmid, pTΔsxyCm, was linearized with NotI and used to transform MIV-competent cells of HS143 as described below. Chloramphenicol-resistant colonies were selected and the *sxy* mutation was confirmed by PCR.

Transformation assays

Transformation with marked chromosomal DNA [from *A. pleuropneumoniae* 4074 *sodC*::kan or from *H. influenzae* MAP7 (NovR)] was assessed on plates, in broth or in MIV medium. Plate transformations were performed as described elsewhere (Bossé *et al.*, 2004). Briefly, 20 μL of bacteria grown to $OD_{600\,nm}$ 0.5 were spotted onto BHI-L plates, with 1 μg DNA added to the cells after 100 min of incubation at 37 °C. Bacteria were scraped from the plates after 4 h at 37 °C, and resuspended and diluted in phosphate-buffered saline before

plating. MIV transformation assays were performed as described elsewhere (Poje & Redfield, 2003): cells growing exponentially in BHI-N or BHI-HN were transferred to MIV medium for 100 min at 37 °C and incubated with 1 μg DNA for 15 min at 37 °C before diluting and plating. Competence development of *A. pleuropneumoniae* HS143 was monitored during growth in BHI-N by removing 1-mL aliquots at different time points, adding 1 μg DNA and incubating for 15 min at 37 °C before adding 1 μg mL^{-1} DNAse I, diluting and plating. In all assays, transformation frequencies were determined as the number of antibiotic-resistant CFU mL^{-1} divided by the total CFU mL^{-1} scored on nonselective agar.

Plasmid-encoded *sxy* expression

The *A. pleuropneumoniae* or *H. influenzae sxy* genes were constitutively expressed from plasmid pMC-Express (Genbank accession no. GQ334689), a broad host-range shuttle vector derived from pMIDG100 (Webb *et al.*, 2001) that contains a chloramphenicol resistance gene and the *A. pleuropneumoniae sodC* promoter situated upstream of a multiple cloning site. The coding sequence for both genes was PCR amplified from the chromosomes of strains 4074 and Rd KW20 and cloned into pGEMT-Easy (Promega). Excision from pGEMT-Easy using ApaI and BstXI enabled directional cloning into the multiple cloning site of pMC-Express, generating plasmids pMCApsxy or pMCHisxy. Expression of each cloned gene was driven by the *A. pleuropneumoniae sodC* promoter, which was previously shown to yield relatively consistent expression under different growth conditions (Langford *et al.*, 1996). These plasmids were transferred to *A. pleuropneumoniae* or *H. influenzae* by conjugation as described previously (Webb *et al.*, 2001).

RNA preparation and reverse transcription (RT)-PCR

To confirm *sxy* expression, RNA was extracted from 1 mL MIV-competent cells using the RNeasy Mini Kit (Qiagen, UK). RT-PCR reactions were performed on serial dilutions of RNA using the OneStep RT-PCR Kit (Qiagen). Both kits were used according to the manufacturer's instructions (Qiagen). Primer pairs GCGGATCCAAAGGAGAAACATA ATGGC/TGCGGTACCGAGATAATCAGAAAGGAACC and GGAGGTTTAATATGAATATAAAGG/TTATTCATCATCTA ATTTTCTATAGC were used to amplify the *A. pleuropneumoniae* and *H. influenzae sxy* genes, respectively.

Results and discussion

To evaluate the distribution of competence in *A. pleuropneumoniae*, the plate-transformation protocol of Bossé *et al.* (2004) was used to screen the reference strains of all 16 serotypes for their ability to transform with marked chromosomal DNA from the serotype 1 strain 4074 (Bossé *et al.*, 2004). As shown in Table 1, most strains produced no transformants at all. This is not likely to be due to the low homology between donor and recipient DNAs, as the 4-kb region flanking *sodC* was 97–100% identical in all *A. pleuropneumoniae* sequenced strains (data not shown). Five of the tested strains (4074, 1421, M62, L20 and 405) transformed at a low frequency (*c.* 10^{-8}), consistent with previously reported values for *A. pleuropneumoniae* (Bossé *et al.*, 2004). In contrast, one strain (HS143) transformed at a 10 000 × higher frequency (10^{-4}). This dramatically higher competence not only makes HS143 a valuable genetic resource for others who study *A. pleuropneumoniae*, but also provided us with the opportunity to reinvestigate competence regulation in this organism.

We have previously shown that treatments used to induce competence in *H. influenzae* and other *Pasteurellaceae* did not increase the competence of *A. pleuropneumoniae* strains 4074 and L20 (Poje & Redfield, 2003; Bossé *et al.*, 2004). To determine whether starvation induces competence in HS143, transformation frequencies were monitored during growth in BHI-N broth culture and upon shift to MIV (Fig. 1). Transformation frequencies in exponential growth were as low as those seen for other transformable *A. pleuropneumoniae* strains, but increased dramatically as cells entered the stationary phase or after transfer to MIV. Competence in this strain therefore appears to be controlled by nutritional signals in the same way as in other transformable *Pasteurellaceae*.

The inefficiency with which *A. pleuropneumoniae* strains other than HS143 transform suggested that they might be defective in some aspect of competence and/or its regulation. The genomes of two of the strains tested in our work (4074 and L20) have been sequenced, and so these were examined for the presence of competence genes. Consistent with our finding that these strains are transformable (Table 1), their genomes contained apparently intact homologues of all genes that *H. influenzae* requires for DNA uptake, with putative CRP-S sequences upstream of their promoters (Table 2A and B). Genome sequences were also available for two *A. pleuropneumoniae* strains not evaluated in our work (JL03 and AP76); however, we had tested other strains of the same serotypes. The results were concordant for each serotype. The sequenced serotype 3 strain (JL03) contained an intact set of competence gene homologues, and the serotype 3 tested in our work (1421) was transformable (Tables 1 and 2). The inverse was true for the serotype 7 strains: the sequenced strain (AP76) had several mutations in its competence genes expected to prevent transformation (Table 2), and the strain tested in this study (WF83) was nontransformable. This suggests that competence may be correlated with serotype in *A. pleuropneumoniae*, although more strains of each serotype should be tested.

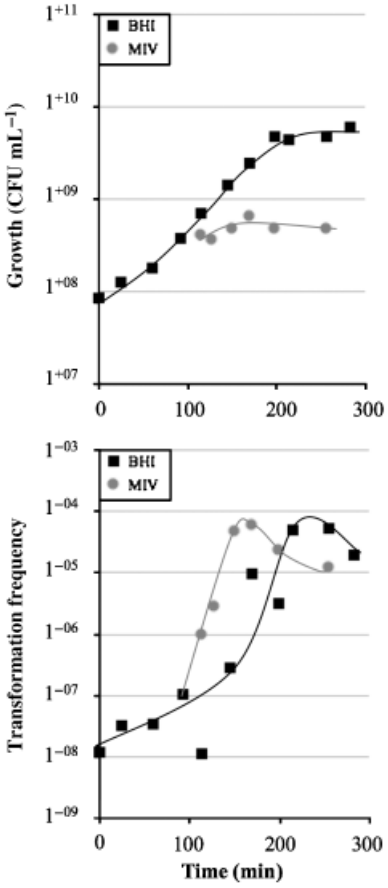

Fig. 1. Growth and competence of *Actinobacillus pleuropneumoniae* HS143. Bacteria were grown in BHI-N either to the stationary phase (black), or with transfer to MIV from BHI at $OD_{600\,nm}$ 0.2 (grey). Viability (a) and transformation (b) were assessed at *c.* 30-min intervals over 300 min of culture. This assay was performed three times and a representative example is shown.

Sxy's role as a positive regulator of competence is well established in *H. influenzae*: a strain lacking *sxy* is not transformable, while strains with mutations that upregulate *sxy* are constitutively competent (Redfield, 1991, 2005; Zulty & Barcak, 1995; Cameron *et al.*, 2008). We confirmed that Sxy plays the same role in *A. pleuropneumoniae* by testing the effect of an *sxy* mutation on competence in strain HS143. Transformation assays of MIV-competent cells with marked chromosomal DNA (*sodC*::kan) showed that the *sxy* knockout strain was nontransformable (Table 1).

We hypothesized that the low competence of the poorly transformable *A. pleuropneumoniae* strains might be due to a defect in their *sxy* homologues. Sequence comparisons ruled out any difference in the *sxy* sequence: all four sequenced *A. pleuropneumoniae* genomes contain identical Sxy homologues that show 26% identity (44% similarity) to *H. influenzae* Sxy, and we found an identical allele in HS143. All four genomes also had identical CRP-S sites at each of the 12 promoters where Sxy acts. These sites (listed in Table 2B) show high identity to the *H. influenzae* CRP-S consensus and retain all bases involved in CRP-DNA binding (Fig. 2).

To investigate whether differences in competence could be caused by differences in *sxy* induction, we tested whether constitutively expressing *sxy* from a plasmid increased the competence of strain 4074. We first compared the activity of *A. pleuropneumoniae* Sxy with that of the well-characterized *H. influenzae* Sxy, by testing complementation of an *H. influenzae sxy* knockout mutation known to completely abolish competence (Zulty & Barcak, 1995) (Fig. 3a). RT-PCR demonstrated that the plasmid-borne *A. pleuropneumoniae sxy* gene was expressed as strongly as the plasmid-borne *H. influenzae sxy* gene, and more strongly than the chromosomal *H. influenzae sxy* gene. In assays of MIV-competent cells with marked *H. influenzae* DNA, this constitutive expression of either *H. influenzae* or *A. pleuropneumoniae sxy* restored high levels of transformation to cells lacking the chromosomal gene, indicating not only that the *A. pleuropneumoniae* gene is functional but also that the extensive sequence divergence between the two species has not altered Sxy's mode of action. Because *H. influenzae* and *A. pleuropneumoniae* represent the two divergent branches of the *Pasteurellaceae* (Redfield *et al.*, 2006), this result confirms that Sxy acts similarly in all *Pasteurellaceae*. Moreover, this validates the usefulness of both plasmids as tools that can be tested for their ability to induce competence in other nontransformable *Pasteurellaceae* strains and species.

We tested whether constitutively expressing both *sxy* genes in *A. pleuropneumoniae* strain 4074 increased its competence. A positive result would mean that cells are failing to become competent because of a failure to induce *sxy*, whereas a negative result would suggest a downstream block to DNA uptake or recombination. RT-PCR showed very low expression of chromosomal *sxy* in strain 4074 and greatly elevated expression of both plasmid-encoded genes (Fig. 3b). However, constitutive expression of either *sxy* gene did not increase transformation.

These experiments therefore confirm that the poor transformability of strain 4074 is not due to an inability to induce *sxy* expression or to a defect in Sxy function. Instead, the problem is likely due to a defect in one or more other components of the competence system, for example a nonfunctional CRP, an inability to induce one or more CRP-S genes, or the poor function of one of more competence proteins.

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Table 2. Competence protein homologues encoded by in *Actinobacillus pleuropneumoniae* genomes. (A) The complete *A. pleuropneumoniae* genome sequences of strains L20 (serotype 5b), 4074 (serotype 1), JL03 (serotype 3) and AP76 (serotype 7) were searched at NCBI (http://www.ncbi.nlm.nih.gov) for homologues of the *Haemophilus influenzae* competence genes. Identities shown are those of each protein sequence to the L20 homologue. (B) Putative CRP-S promoter sites were identified manually

H. influenzae	*A. pleuropneumoniae*						
(A)							
Regulators:							
Rd KW20	L20	4074	ID to L20	JL03	ID to L20	AP76	ID to L20
Sxy (HI0299)	APL_1758	Aple02002132	100	APJL_1793	100	APP7_1843	100
CRP (HI0299)	APL_1965	Aple02000640	100	APJL_2012	100	APP7_2053	100
Competence proteins:							
Rd KW20	L20	4074	ID to L20	JL03	ID to L20	AP76	ID to L20
ComA (HI0439)	APL_0196	Aple02001014	100	APJL_0197	99	APP7_0199	99
ComB (HI0438)	APL_0197	Aple02001013	100	APJL_0198	100	APP7_0200	100
ComC (HI0437)	APL_0198	Aple02001012	100	APJL_0199	99	APP7_0201	99
ComD (HI0436)	APL_0199	Aple02001011	100	APJL_0200	100	APP7_0202	98
ComE (HI0435)	APL_0200	Aple02001010	100	APJL_0201	100	APP7_0203	100
ComF (HI0434)	APL_2004	Aple02001940	100	APJL_2054	100	APP7_2091	100
PilA (HI0299)	APL_0880	Aple02000139	100	APJL_0892	99	APP7_0939	99
PilB (HI0298)	APL_0879	Aple02000138	100	APJL_0891	99	APP7_0938	99
PilC (HI0297)	APL_0878	Aple02000137	99	APJL_0890	99	APP7_0937	99
PilD (HI0296)	APL_0877	Aple02000136	100	APJL_0889	99	APP7_0936	99
Rec-2 (HI0061)	APL_0766	Aple02000700	100	APJL_0768	99	APP7_0827	99
ComE1 (HI1008)	APL_1406	Aple02002116	100	APJL_1431	99	APP7_1533	100
PulG/ComN (HI0938)	APL_1888	Aple02000828	100	APJL_1931	99	APP7_1975	100
PulJ/ComO (HI0939)	APL_1887	Aple02000829	100	APJL_1930	100	APP7_1974	100
ComP (HI0940)	APL_1886	Aple02000830	100	APJL_1929	99	APP7_1973	100
ComQ (HI0941)	APL_1885	Aple02000831	100	APJL_1928	98	APP7_1972	100
DprA (HI0985)	APL_1712	Aple02001929	100	APJL_1744	96	APP7_1772	98
ComM (HI1117)	APL_1747	Aple02001780	100	APJL_1782	99	APP7_1816/1832*	–
HI0659	APL_1357	Aple02000791	100	APJL_1375	100	APP7_1409	100
HI0660	APL_1358	Aple02000792	100	APJL_1376	100	†	100
HI0365	APL_1274	Aple02001576	100	APJL_1284	98	APP7_1325‡	–
PilF (HI0366)	APL_1273	Aple02001575	100	APJL_1285	99	APP7_1323	100
RadC (HI0952)	APL_1970	Aple02000635	100	APJL_2017	100	APP7_2058	100
Ssb (HI0250)	APL_0783	Aple02000723	100	APJL_0787	100	APP7_0845	100

Gene name	Species	CRP-S site
(B)		
comA	*H. influenzae*	TTTTGCGATCCGCATCGTAAAA
	A. pleuropneumoniae	TTTTGCGATCTTCATCGAAAAA
comF	*H. influenzae*	§
	A. pleuropneumoniae	TTTTCCGATCCGTATCGCAAAA
pilA	*H. influenzae*	TTTTGCGATCAGGATCGCAGAA
	A. pleuropneumoniae	TTTTGCGATACGGATCGCAGAA
rec-2	*H. influenzae*	TTTTACGATATGGATCGCAAAA
	A. pleuropneumoniae	TTTTGCGATCAGGATCGAAGAA
comE1	*H. influenzae*	TTTTGCGATCGAGATCGCAAAA
	A. pleuropneumoniae	TTTCTCGATCCTGATCGCAAAA
pulG/comN	*H. influenzae*	CTTTGCGATACAGATCGCAAAA
	A. pleuropneumoniae	TTTTGCGATCAAGATCGAATAA
dprA	*H. influenzae*	TTTTGCGATCTGCATCGCAAAA
	A. pleuropneumoniae	TTTTGTGATCTCAATCGAAAAA
comM	*H. influenzae*	TTTTGCGATCTAGATCGCAAAA
	A. pleuropneumoniae	TTTTGCGATCCTGATCGAGAAA
HI0659	*H. influenzae*	TTTTGCGATCTAGATCGAAAGA
	A. pleuropneumoniae	TTACGCGTTTTCATTCAAAAGG
HI0365	*H. influenzae*	ATTTGCGATCTAGATCGCAAAA
	A. pleuropneumoniae	TTTTGCGATCTTGATCGCAAAC

Table 2. Continued.

Gene name	Species	CRP-S site
radC	*H. influenzae*	TTTTACGATATGCATCGCAGAT
	A. pleuropneumoniae	TTTTGCGATCCGTGTCGAAAAAA
ssb	*H. influenzae*	TTTTGCGATCATTATCGCATAT
	A. pleuropneumoniae	AATGTTTTTTATTATCGCATAT

Sequences shown for *Haemophilus influenzae* are those from strain Rd KW20. Sequences shown for *Actinobacillus pleuropneumoniae* were identical in all four sequenced genomes. Differences between the *H. influenzae* and *A. pleuropneumoniae* are highlighted in grey.
*Large insertion in the AP76 *comM* gene.
†Present in AP76 and 100% identical to L20 gene, but not annotated.
‡Truncated because of single base pair insertion.
§The *comABCDEF* forms one operon in *H. influenzae* but is split in *A. pleuropneumoniae*, each with its own predicted CRP-S site.

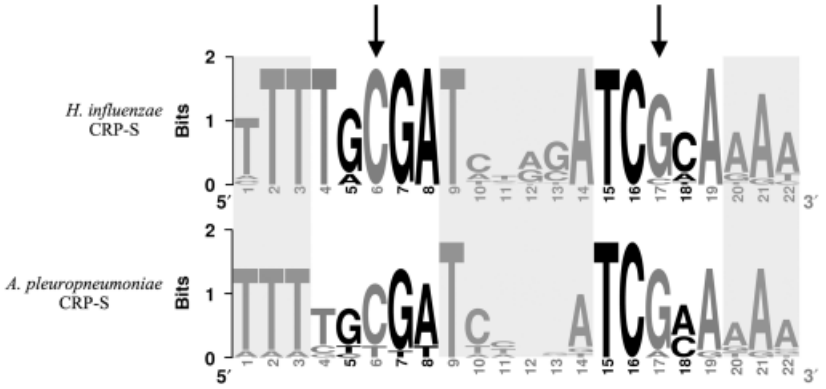

Fig. 2. Sequence logos for CRP-S sites of *Haemophilus influenzae* (top) and *Actinobacillus pleuropneumoniae* (bottom). Sequence logos were generated with WebLogo (http://weblogo.berkeley.edu/logo.cgi) (Crooks *et al.*, 2004), using 13 *H. influenzae* sites or 12 *A. pleuropneumoniae* sites. Bases important for CRP-DNA binding are shown in black, and the positions characteristic of CRP-S sites (C_6/G_{17}) are shown with arrows. Noncore positions are shaded.

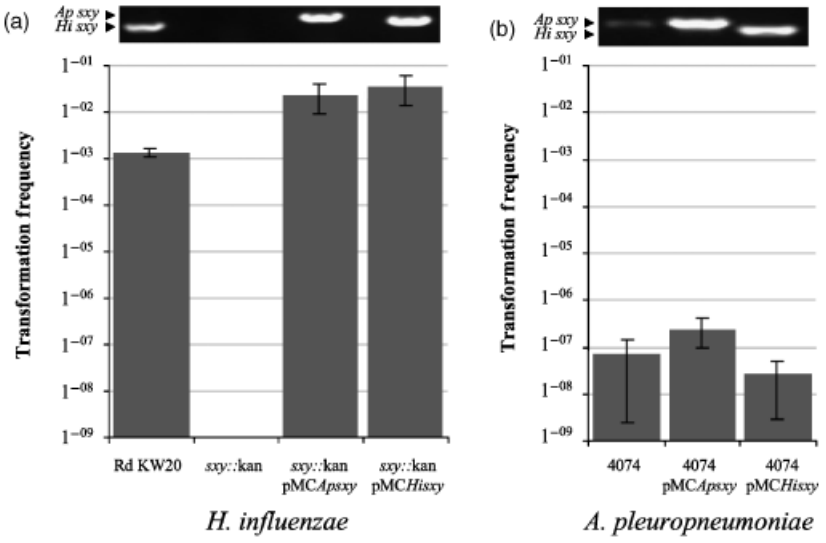

Fig. 3. Effect of plasmid-borne *sxy* expression on competence after induction with MIV medium. RT-PCR was used to monitor the expression of *Haemophilus influenzae* (665 bp) or *Actinobacillus pleuropneumoniae* (839 bp) *sxy* genes using 0.01 ng RNA template for each strain. The resulting DNA products are shown above each bar. Bars represent transformation frequencies determined over four replicate assays; error bars show SD. (a) In *H. influenzae*: 1, Rd KW20 (parent strain); 2, *sxy*::kan; 3, *sxy*::*kan* pMC*Apsxy*; 4, *sxy*::*kan* pMC*Hisxy*. (b) In *A. pleuropneumoniae*: 5, 4074; 6, 4074 pMC*Apsxy*; 7, 4074 pMC*Hisxy*.

Authors' contribution

J.T.B. and S.S. contributed equally to this work.

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