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Surface-layer (S-layer) of human and animal *Clostridium difficile* strains and their behaviour in adherence to epithelial cells and intestinal colonization

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Clostridium difficile is a frequent cause of severe, recurrent post-antibiotic diarrhoea and pseudomembranous colitis. The surface layer (S-layer) is the predominant outer surface component of *C. difficile* which is involved in pathogen–host interactions critical to pathogenesis. In this study, we characterized the S-layer protein A (SlpA) of animal and human strains belonging to different PCR-ribotypes (PR) and compared the *in vitro* adherence and *in vivo* colonization properties of strains showing different SlpA variants. Since each SlpA variant has been recently associated with an S-layer cassette, we were able to deduce the cassette for each of our strains. In this study, an identity of 99–100 % was found among the SlpA of isolates belonging to PR 012, 014/020, 045 and 078. One exception was the SlpA of a poultry isolate, PR 014/020, which showed 99 % identity with that of strain 0160, another PR 014/020 which contains an S-layer cassette 6. Interestingly, this cassette has also been found in a PR 018 strain, an emerging virulent type currently predominant in Italy. Five other SlpA variants (v014/020a–e) were identified in strains PR 014/020. *In vitro* adherence assays and *in vivo* colonization experiments were performed on five PR 014/020 strains: human 1064 (v014/020e), human 4684/08 (v014/020b), human IT1106 (v078a), poultry P30 (v014/020d) and poultry PB90 (v014/020b) strains. Adhesion assays indicate that *C. difficile* strains vary in their capacity to adhere to cells in culture and that adhesion seems to be independent of the SlpA variant. Colonization properties were assessed *in vivo* using a dixenic mouse model of colonization. The kinetics of faecal shedding and caecal colonization were similar when human 4684/08 (v014/020b) strain was compared with human 1064 (v014/020e) and poultry PB90 (v014/020b) strain. In contrast, poultry P30 (v014/020d) strain outcompeted both human 4684/08 (v014/020b) and IT1106 (v078a) strains and its adherence to caeca at day 7 was significantly higher. The peculiar characteristics of *C. difficile* P30 seem to advantage it in colonizing the intestinal mice niche, increasing its ability to compete and adapt. The results obtained underline the need of an increased attention to the genetic evolution of *C. difficile* to prevent and limit the consequences of the emergence of increasingly virulent strains.

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Abbreviations: CDI, *C. difficile* infection; PR, PCR-ribotype; S-layer, surface layer; SLP, S-layer protein.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the *slpA* genes coding for the new SlpA variants v014/020a, v014/020c, v014/020d, v014/020e, v045a, v045b, v045c and v027a are HF569014–HF569021, respectively.

Two supplementary figures are available with the online version of this paper.

INTRODUCTION

Clostridium difficile is a Gram-positive anaerobic bacterium that colonizes the intestine of humans and animals. Antibiotic exposure, advanced age and hospitalization are the major factors associated with *C. difficile* infection (CDI) (Kelly *et al.*, 1994; Rupnik *et al.*, 2009). CDI can range from mild diarrhoea, moderately serious disease, to severe life-threatening pseudomembranous colitis (Bartlett., 2006).

Although TcdA and TcdB contribute directly to CDI-associated lesions of the intestinal mucosa (Thelestam & Chaves-Olarte, 2000; Rupnik & Just, 2006), other factors appear to be involved in pathogenesis of *C. difficile*, by contributing to the colonization and to genesis of mucosal damage. The surface layer (S-layer) is the predominant outer surface component of *C. difficile*. It is organized in two superimposed crystalline arrays and composed of two different proteins, the high molecular weight protein (HMW SLP) and the low molecular weight protein (LMW SLP) (Calabi *et al.*, 2001; Cerquetti *et al.* 2000). These proteins are encoded as a single precursor (SlpA) by the *slpA* gene. The SlpA undergoes a post-translational cleavage giving the two mature peptides. The HMW SLP derives from the C-terminal region and it is relatively conserved among *C. difficile* strains, whereas the LMW SLP derives from the N-terminal region and its sequence is more highly variable (Calabi *et al.*, 2002).

The S-layer is involved in pathogen–host interactions critical to pathogenesis. It has been demonstrated that the SLPs facilitate *C. difficile* adhesion to cultured cell lines and adhere to both gastrointestinal tissues and several extracellular matrix components (Takumi *et al.*, 1991; Calabi *et al.*, 2002). Chemical removal of the SLPs or neutralization with anti-SLP Fab fragments was shown to abolish adherence of *C. difficile* to human HeLa or mouse 929 cells (Takumi *et al.*, 1991). Furthermore, the LMW SLP is a predominant *C. difficile* surface antigen and displays variability to escape host immune response. However, a strong serum IgG response has been observed in sera from convalescent patients who have had CDI (Pantosti *et al.*, 1989). For these reasons, the S-layer is among the main potential candidates for multicomponent vaccines against CDI.

Even though SlpA amino acid sequence has been found highly conserved in some human *C. difficile* isolates of the same PCR-ribotype (PR) (Eidhin *et al.*, 2006; Spigaglia *et al.*, 2011), it has been recently demonstrated to be heterogeneous in strains belonging to the same type (PR) (Dingle *et al.*, 2013). The genetic diversity also involved *cwp66* and *secA* genes. These genes together with *slpA* are contained in a 10 kb genetic region denominated the S-layer cassette. Twelve different S-layer cassette variants have been identified and these cassettes can randomly associate with different genotypes by homologous recombination events (S-layer switching).

While the SlpA of human strains has been investigated in different studies (Eidhin *et al.*, 2006; Spigaglia *et al.*, 2011), the SlpA of animal strains has not been investigated in such detail yet, even though many PRs are common to humans and animals and transmission of particular *C. difficile* types between humans and animals is likely to occur (Squire & Riley, 2012; Hensgens *et al.*, 2012; Janezic *et al.*, 2012). PR 014/020 and 078 are among the types most frequently found in both humans and animals (Hensgens *et al.*, 2012; Freeman *et al.*, 2010; Janezic *et al.*, 2012). PR 014/020 is the predominant PR isolated in European hospitals and is found in many different animal species (Bauer *et al.*, 2011; Janezic *et al.*, 2012). PR 078 is an emergent PR that is recognized as a cause of severe CDI both in hospitals and in the community (Bauer *et al.*, 2011). Furthermore, there is evidence suggesting a possible zoonotic transmission of strains PR 078 between humans and animals (Goorhuis *et al.*, 2008), even if definitive proof is still missing.

The aim of this study was to characterize the SlpA of *C. difficile* from several PRs which include animal and human strains and to compare the *in vitro* adherence to epithelial cells and *in vivo* colonization properties of strains of the same PR but with different SlpA variants.

METHODS

***C. difficile* strains and typing.** The majority of *C. difficile* strains were selected from the large collection at the Institute of Public Health Maribor. Strain IT1106 was selected from the Istituto Superiore di Sanità collection. The strains used in this study belonged to PRs present in humans and different animal species (PR 014/020, 045, 012, 027 and 078). The PR was again confirmed by PCR-ribotyping using the method of Bidet *et al.* (1999). Toxinotype was determined before using the method described by Rupnik (2010).

Strains were cultured under anaerobic conditions on *Brucella* agar plates containing vitamin K1 (0.5 mg l^{-1}), haemin (5 mg l^{-1}) and 5 % defibrinated sheep red blood cells and in brain–heart infusion (BHI) broth (Oxoid) for DNA extraction.

Antibiotic susceptibility. MICs of erythromycin, clindamycin, moxifloxacin, tetracycline and rifampicin were determined by Etest (AB Biodisk) on *Brucella* agar (BA) plates containing vitamin K1 (0.5 mg ml^{-1}), haemin (5 mg ml^{-1}) and 5 % defibrinated sheep red blood cells. Quality control strains used for susceptibility testing included *Bacteroides thetaiotaomicron* ATCC 29741. The breakpoints were 8 mg l^{-1} for erythromycin, clindamycin and moxifloxacin and 16 mg l^{-1} for tetracycline, in accordance with the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) for anaerobic bacteria (CLSI, 2007). Since the breakpoint for rifampicin is not provided for anaerobes, the value of 4 mg l^{-1} was used, in accordance with the CLSI interpretive categories approved for *Staphylococcus aureus* (CLSI, 2008).

Sequence analysis. SlpA sequence analysis was performed as already described for PR 012, 014/020, 027 and 078 (Spigaglia *et al.*, 2011). We used the same primers designed for the *slpA* gene of PR 012 to amplify the *slpA* of PR 045. PCR conditions were: 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 46–48 °C (depending on the melting temperature of the primers) for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 5 min. Nucleotide and amino acid sequence analysis was performed using Lasergene version 8.0 software (DNASTAR), the National Center for Biotechnology Information BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and the European Bioinformatics Institute CLUSTAL W server (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The reads covering the *slpA* gene of the isolates analysed by Dingle *et al.* (2013) for the S-layer cassette were assembled using Geneious Read Mapper (Biomatters) (Kearse *et al.*, 2012). The genomic reads are available at http://www.ebi.ac.uk/ena/data/view/ERP001417.

The human SlpA reference sequences used in amino acid sequence comparison analysis were AAZ05975 (strain HPA R13550),

Table 1. Characteristics of the *C. difficile* strains analysed in this study

Strain	Source	PCR-ribotype	Toxinotype	SlpA variant
CD168	Dog	014/020	0	v014/020a
PSOB22-3	Dog	014/020	0	v014/020a
PB90	Poultry	014/020	0	v014/020b
MSO2	Cat	014/020	0	v014/020c
P30	Poultry	014/020	0	v014/020d
1064	Human	014/020	0	v014/020e
4684/08	Human	014/020	0	v014/020b
B-806/1	Pig	045	V	v045a
369-2/2I	Calf	045	V	v045b
SE866	Human	045	V	v045c
SE881	Human	045	V	v045c
ZZV07-541	Calf	027	III	v027a
ZZV07-22	Calf	027	III	v027b
CD167	Dog	012	0	v012a
5 3897-2/2 IV	Calf	012	0	v012a
ZZV07-542	Pig	078	V	v078a
5 347-2 III	Calf	078	V	v078a
IT1106	Human	078	V	v078a

AAZ05984 (strain HPA R12885), YP_006199863 (strain BI1), for PR 012, 014/020 and 027, respectively. Both AAZ05994 (strain HPA R13540) and ZP_05402187 (strain QCD 23m63) were used as reference sequences for PR 078. All these sequences were already deposited in GenBank. No previously deposited reference SlpA sequences were available for PR 045.

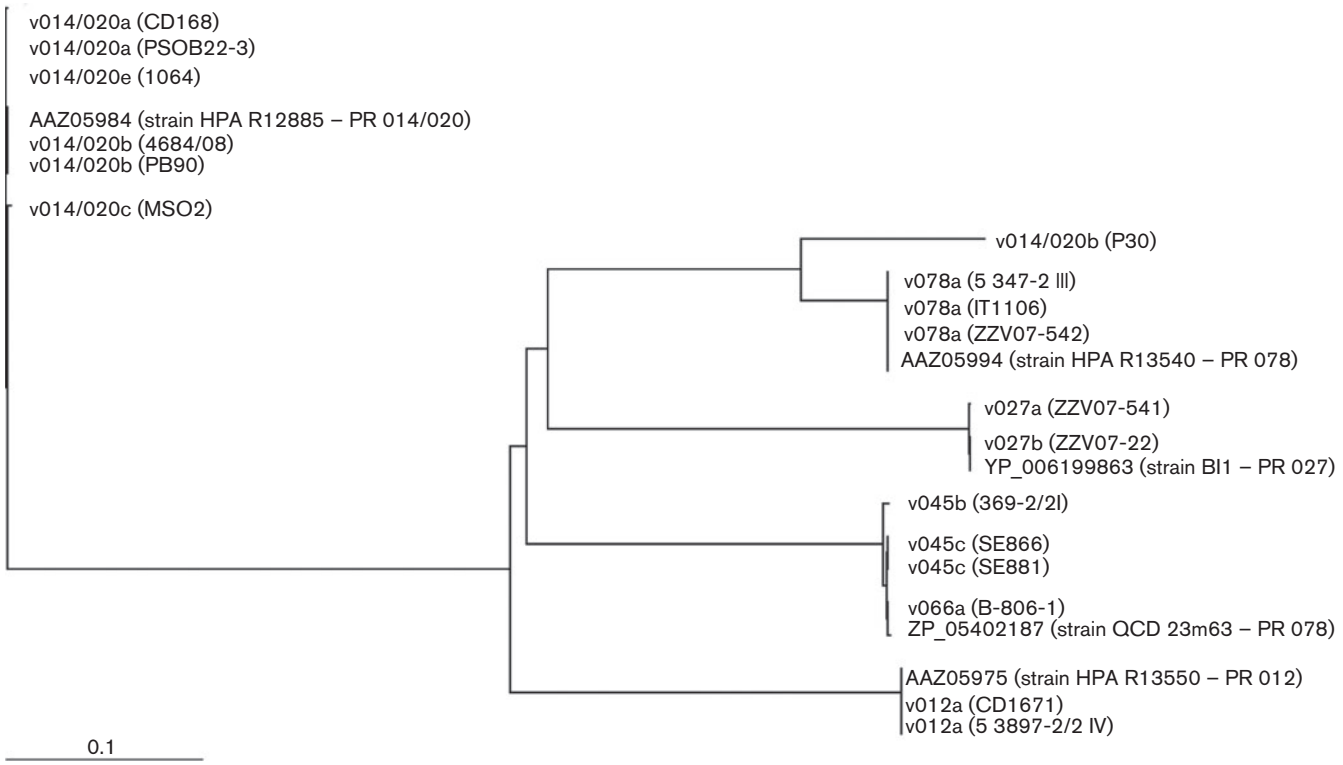

Fig. 1. Phylogenetic tree based on the alignment of the S-layer protein A amino acid sequences of the 18 *C. difficile* strains analysed in this study with the reference sequences for PCR-ribotypes PR 012, 014/020, 027 (GenBank accession numbers AAZ05975, AAZ05984, YP_006199863) and 078 (AAZ05994 and ZP_05402178). The phylogram was generated by using TreeView version 1.6.6. The branch lengths are scaled in proportion to the extent of the change per position, as indicated by the bar.

Table 2. Antibiotic resistance of selected *C. difficile* strains

ERY, erythromycin; MXF, moxifloxacin; CLI, clindamycin; RIF, rifampicin.

Strain	Source	SlpA variant	MIC (mg l^{-1})			
			ERY	MXF	CLI	RIF
PB90	Poultry	v014/020b	1	0.5	3	⩽0.002
P30	Poultry	v014/020d	3	1.5	8	⩽0.002
1064	Human	v014/020e	3	⩾256	2	⩽0.002
4684/08	Human	v014/020b	⩾256	1.5	⩾256	⩽0.002
IT1106	Human	v078a	⩾256	0.38	3	⩽0.002

***In vitro* adhesion assay.** *In vitro* adhesion was performed on Caco-2 cells as already described (Cerquetti *et al.*, 2002). Briefly, cells were cultured in pre-reduced minimum essential medium with Earle's salts (MEM) (Gibco-BRL, Life Technologies). Monolayers were used at 3 (non-confluent monolayers) and 15 days (post-confluent monolayers) after seeding. The monolayers analysed at 15 days were also tested after a treatment with 0.1 mM EGTA, used to disrupt intercellular junctions, in S-MEM (Gibco-BRL, Life Technologies). Ten millilitres of overnight cultures of different *C. difficile* strains were centrifuged, the pellet was washed three times with PBS, adjusted to OD_{600} 1.0, diluted 1 : 10 (1×10^8 bacteria ml^{-1}) in MEM or S-MEM plus EGTA and added to each tissue-culture plate well. After 1.5 h of incubation in anaerobic conditions the non-adherent bacteria were removed by washing with PBS and the bound bacteria were detached by adding saponin (1 %). Serial dilutions were plated and c.f.u. were counted after 48 h of incubation. To express the results as the number of adherent bacteria per cell, Caco-2 cells from non-infected monolayers were collected by trypsinization and counted in parallel (Barketi-Klai *et al.*, 2011). Caco-2 cells were counted, after 3 and 15 days, using a Burker chamber. Control wells, where *C. difficile* cells in MEM or S-MEM were added in the absence of any Caco-2 cells, were also included. Results for each assay condition were obtained as the mean of three independent assays. Statistical analyses were performed using the Mann–Whitney test with GraphPad Prism software. A *P*-value <0.05 was considered significant.

***In vivo* competition experiments.** C3H/HeN germ-free mice from CDTA (CNRS Orléans, France) were used. *C. difficile* was grown in BHI broth at 37 °C in an anaerobic chamber overnight. Bacteria were harvested by centrifugation, suspended in PBS and diluted to 2×10^7 cells ml^{-1} after counting by a Malassez haemocytometer. Real density was determined subsequently from viable counts of c.f.u. Groups of 6-week-old male mice (n=6) were orally challenged with 0.5 ml of bacterial suspension containing a mixture of two different strains in equal amounts and monitored for up to 7 days. Strain pairs were selected according to PR, Slp type and difference in resistance to at least one antibiotic (to enable subsequent differentiation by culture after challenge). Faecal samples were collected every day from each mouse. They were weighed and diluted in PBS in order to obtain a concentration of 10 mg ml^{-1}. The number of bacteria present in faeces was determined by using serial dilutions in PBS and by seeding them on BHI agar and BHI agar supplemented with 10 μg ml^{-1} erythromycin, according to the erythromycin resistance of the selected strains. Seven days after *C. difficile* challenge, mice were sacrificed and dissected in order to obtain the entire caecum of each mouse. Each caecum was washed eight times by gentle shaking in PBS buffer, weighed and diluted in PBS to a final concentration of 10 mg ml^{-1}. The caecum was then homogenized with an ultraTurrax apparatus (IKA-Labortechnik) for 1 min at 13 500 r.p.m. Serial dilutions were seeded and cultured in BHI with and without erythromycin. C.f.u. were counted after 48 h of incubation. Statistical analyses were performed using the Mann–Whitney test with GraphPad Prism software. A *P*-value <0.05 was considered significant.

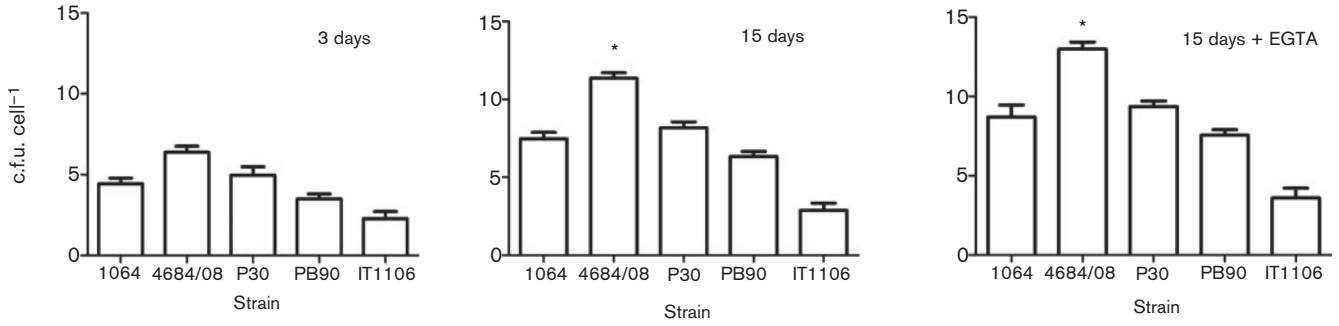

Fig. 2. Adherence of *C. difficile* to intestinal epithelial cell lines. Bacteria were incubated with monolayers of Caco-2 cells. Monolayers at 3 days were considered undifferentiated, and monolayers at 15 days fully differentiated. Cell binding was measured by the bacterial attachment assay as described in Methods. Results are presented as the number of bound bacteria (c.f.u.) per cell. Each assay was performed in triplicate. The values presented are means ± SD of three independent experiments. Statistically significant differences are indicated by * for $P<0.05$.

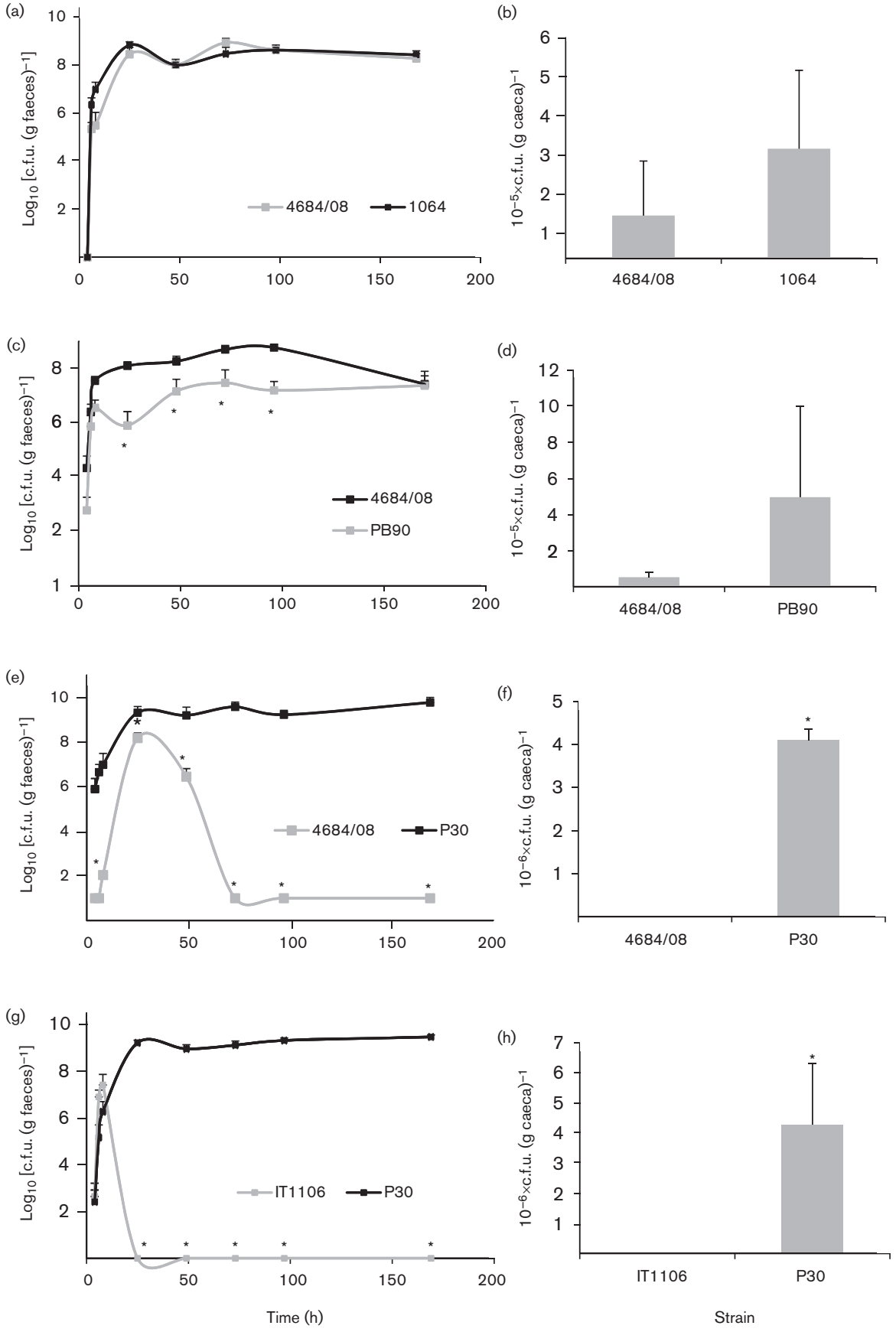

Fig. 3. Intestinal colonization of dixenic mice by *C. difficile* strains (competition assays). Kinetics of intestinal faecal shedding by (a) human 1064 (v014/020e) vs human 4684/08 (v014/020b), (c) human 4684/08 (v014/020b) vs poultry PB90 (v014/020b), (e) human 4684/08 (v014/020b) vs poultry P30 (v014/020d), and (g) human IT1106 (v078a) vs poultry P30 (v014/020d) pairs of strains. (b, d, f, h) Caecal colonization by each indicated pair of strains. Statistically significant differences ($P<0.05$) are indicated by *. Error bars indicate SD.

RESULTS AND DISCUSSION

PR 014/020 *C. difficile* strains display a high SlpA variability

Eighteen *C. difficile* strains (13 from animals and five from humans), belonging to different PRs (012, 014/020, 027, 045, 078), were analysed. The characteristics of these strains are shown in Table 1 and the SlpA phylogenetic analysis in Fig. 1. The *slpA* genes and the derived amino acid sequence alignments are shown in Figs S1 and S2 (available in JMM Online), respectively.

In general, five different SlpA variants were identified among PR 014/020 strains, three among PR 045 strains, two among PR 027 strains and only one amino acid sequence type among strains belonging to PR 012 and 078 (Table 1). We arbitrarily named the variants with a v followed by the PR and a lower case letter (v014/020a, v014/020b, etc.).

In this study, the SlpA of the strains belonging to the same type were highly conserved (99–100 % identity), independently of the origin, except for the poultry strain P30, belonging to PR 014/020 (Fig. 1).

Since SlpA variants have been recently associated with specific S-layer cassettes (Dingle *et al.*, 2013), the SlpA found in our strains were compared with those found in the cited study to deduce the possible associated cassette.

Surprisingly, the SlpA of strain P30 (v014/020d) showed a low identity (50 %) with the amino acid sequences of the other PR 014/020 strains but it has 99 % identity with that of *C. difficile* 0160, an isolate examined by Dingle e*t al.* (2013), and typed as PR 014. Thus, it is very probable that P30 has an S-layer cassette 6 as does 0160. This cassette seems to be not so widespread among *C. difficile* isolates but, interestingly, it has also been found in a PR 018 strain, an emerging virulent type (Bauer *et al.*, 2011). P30 SlpA also shows a high identity (87 %) with the amino acid sequences of the PR078 strains analysed in this study (Fig. 1). Regarding the SlpA variants found in the other PR014/020 strains, v014/020b showed 100 % identity with the reference amino acid sequence for this type (GenBank accession number AAZ05984 – strain HPA R12885), whereas v014/020a, v014/020c and v014/020e had 99 % identity with this amino acid sequence. Probably, the S-layer cassette present in these strains is type 10; in fact their SlpA have an identity ranging between 99 and 100 % with that of *C. difficile* 0098 (a PR 020 isolate) or 6325 (a PR 014 isolate), both showing this type of cassette.

Interestingly, the three SlpA variants identified in PR 045 strains had 99 % identity with that of strain 2490, the only PR 045 strain included in the study of Dingle *et al.* (2013), which contains a cassette 8. These variants also showed 99 % identity with the SlpA of strain QCD 23m63 (GenBank accession number ZP_05402187). This strain is typed as PR 078 but its SlpA is only 59 % identical to those of the other PR078 strains. Further analysis will be necessary to confirm the PR of this strain and to characterize its S-layer cassette.

The SlpA of the PR 078 strains examined in this study were identical to both the SlpA of the reference amino acid sequence (GenBank accesion number AAZ05994 – strain HPA R13540) and that of *C. difficile* 1729, the only PR078 strain investigated for the S-layer cassette. Therefore, the PR 078 strains we examined could have the same S-layer cassette as that strain. This cassette, denominated H2/6, seems to have recently originated from cassette 2 and 6 by recombination.

Even though the SlpA and the associated cassette are different, genomic analyses have demonstrated that isolates 2490 (PR 045) and 1729 (PR 078) belong to the same clade (Dingle *et al.*, 2013). Typing results and phylogenetic analysis, performed on the PCR-ribotyping patterns produced by different PR 045 and PR 078 isolates, confirmed that these strains belong to toxinotype V and have a very high percentage of similarity (>87 %) (data not shown).

The SlpA v027b and v027a were 100 and 99 % identical to the reference sequence (GenBank accession number YP_006199863 – strain BI1), respectively. The same percentage identities were observed when the two variants were compared with the SlpA of isolate 0090, the only PR 027 strain included in the study of Dingle *et al.* (2013), which shows a cassette 4.

All PR 012 strains showed a SlpA identical to the respective reference sequence (GenBank accession number AAZ05975 – strain HPA R13550). This amino acid sequence has 100 % identity with the SlpA of isolate 2479, containing an S-layer cassette 8.

The results indicate that P30 has a SlpA with a high identity with those of the PR 078 strains investigated in this study and suggest that it probably contains a cassette 6. Both the *slpA* and the *secA2* genes of this cassette have been demonstrated to be homologous to those of cassette H2/6 of PR 078.

For these intriguing characteristics, both *in vitro* adhesion and *in vivo* competitive assays were performed on P30 in comparison with other strains PR 014/020 showing different SlpA variants (Table 2).

Bacterial cell adhesion is independent from the SlpA type and the origin of strains

In vitro adherence assays to CaCo-2 cells demonstrated an adhesion significantly higher for the human strain 4684/08 (v014/020b) compared with the other strains, except when it was compared with the poultry strain P30 (v014/020d) at day 3 ($P<0.05$) (Fig. 2). The ability to adhere *in vitro* to the intestinal epithelial cells seems to be independent from the *C. difficile* strain origin and from the SlpA type. This is not surprising since adherence is a multifunctional event and many surface elements are involved in this process (Barketi-Klai *et al.*, 2011; Hennequin *et al.*, 2001; Tasteyre *et al.*, 2001; Waligora *et al.*, 2001). Nonetheless, the human 4684/08 and the poultry P30 strains seem to adhere better to differentiated cells organized in monolayers and to cells not completely differentiated, respectively. Further studies on surface components are necessary to clarify the results obtained.

Poultry v014/020d variant strain outcompetes human strains in intestinal colonization of mice

In order to assess the capacity of animal and human strains to colonize mice, kinetics of faecal shedding and caecal colonization were studied in the dixenic mouse model. Kinetics of faecal shedding allow measurement of the intestinal multiplication of a strain, while caecal colonization is a measure of the capacity of the strain to interact with the intestinal epithelium. For each experiment, faeces were collected at specific times during a week and caeca were obtained by sacrificing mice 7 days post-challenge.

A different pattern of resistance is required for the strains used in each *in vivo* competition assay. The antibiotic susceptibility profiles are summarized in Table 2.

We carried out competition assays in which mice were simultaneously challenged with both an erythromycin-resistant and an erythromycin-sensitive strain according to antibiotic resistance profiles. The following pairs of strains were tested: human 4684/08 (v014/020b) strain versus human 1064 (v014/020e), poultry PB90 (v014/020b) and poultry P30 (v014/020d) strains, respectively. In addition, the poultry P30 strain was tested in competition with a human PR 078 strain (IT1106, v078a) because of 87 % SlpA identity between v014/020d and PR 078.

The kinetics of faecal shedding and caecal colonization were similar when human 4684/08 (v014/020b) strain was compared with human 1064 (v014/020e) strain (Fig. 3a, b). The same human strain (4684/08) showed a better ability to shed during the first 5 days after challenge than the poultry PB90 (v014/020b) strain (Fig. 3c). After 120 h post-challenge the shedding rate became similar for these two strains. Even if a lower rate of caecal colonization at day 7 post-infection was observed for the human 4684/08 (v014/020b) strain in this model, this difference was not statistically significant (Fig. 3d).

In contrast, poultry P30 (v014/020d) strain outcompeted both human 4684/08 (v014/020b) (Fig. 3e) and IT1106 (v078a) (Fig. 3g) strains and its adherence to caeca at day 7 was significantly higher (Fig. 3f, h).

In order to exclude a variability of growth rate of each strain that could explain the differences observed in animal competition experiments, the same pairs of strains were tested in liquid (BHI) co-cultures at 4, 6, 8, 24 and 48 h. As expected, similar growth curves were obtained for each tested strain and no outcompetition was observed between strains. In particular, the P30 strain did not outcompete the other strains unlike in the mouse model (data not shown).

Altogether, these results indicate that the poultry P30 (v014/020d) strain exhibits an ecological advantage for colonizing the intestinal animal niche. Interestingly, this strain has a combination of properties of the two currently most prevalent strains in humans and in animals (Janezic *et al.*, 2012; Bauer *et al.*, 2011). It displays ribotyping profile PR 014/020 and SlpA type very similar to that of strains PR 078.

CONCLUSIONS

In this study, a first characterization and comparison between the SlpA of human and animal *C. difficile* strains was performed. The S-layer is involved in multiple functions and plays a key role in the establishment within the host and the competitiveness of *C. difficile* strains. In this study, the SlpA of the strains belonging to the same PR was highly conserved, with a 99–100 % identity, independent of the source (animal or human), even though it is known that strains of the same type do not always show the same SlpA sequence and S-layer cassette (Dingle *et al.*, 2013). We also observed that *C. difficile* strains vary in their capacity to adhere to cells in culture and that adhesion seems to be independent of the SlpA variant. Interestingly, comparative sequence analysis suggests that the SlpA variant of the P30 poultry strain PR 014/020 could be associated with cassette 6, a cassette also found in a PR 018 strain, an emerging highly virulent type currently predominant in Italy (Bauer *et al.*, 2011). We used gnotoxenic mouse as a model to evaluate the capability of strains to adapt to a given ecological niche. The peculiar characteristics of *C. difficile* P30 seem to advantage this strain in colonizing the mouse intestinal niche. Certainly, the SlpA and the whole S-layer cassette have an important role in the competitiveness and adaptability of *C. difficile* strains, so further studies will be necessary to better understand their function.

The genetic variability is becoming increasingly important for the emergence of virulent strains able to adapt to different environments and hosts. This study underlines the need for increased attention to and careful analysis of strains of different origin to better understand the evolution of *C. difficile* and to prevent and limit the consequences of the emergence of increasingly virulent strains.

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