BACTERIAL CHARACTERISATION

Subspecific differentiation of *Burkholderia cepacia* isolates in cystic fibrosis

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Thirty clinical isolates of *Burkholderia cepacia* from cystic fibrosis (CF) patients in the UK and Denmark were characterised, together with other clinical isolates and laboratory strains of *B. cepacia*, *B. gladioli* and *B. vietnamiensis*. Outer-membrane protein (OMP) profiles were determined, and the organisms were typed genotypically by pulsed-field gel electrophoresis after DNA restriction analyses with *Xba I* and *Dra I*. This latter method revealed four clusters among the clinical isolates studied; one of these contained isolates of the UK and intercontinental CF epidemic lineage ET12, a cluster which appeared to contain three subtypes. Each of the four clusters appeared less closely related to laboratory strains of *B. cepacia* than were laboratory strains of *B. vietnamiensis*, but more closely related to both these species than to *B. gladioli*. Two types of OMP profile were distinguished among the clinical isolates and strains, and were designated A and B. In type A isolates the major proteins had mol.wts of 39, 27 and 18 kDa. Type B strains additionally contained a group of proteins in the size range 80–90 kDa, although detection of these depended upon the conditions for sample denaturation. In most cases, the OMP type correlated with the genotype, suggesting that examination of OMPs might be of value in the initial characterisation of isolates.

Introduction

Infection with *Burkholderia cepacia* is a recognised complication in the progression of pulmonary disease in cystic fibrosis (CF) patients [1, 2]. The organism is also a pathogen in individuals immunocompromised by chronic granulomatous disease (CGD) and AIDS [3, 4]. *B. cepacia* shows considerable metabolic diversity, but is less prevalent in the environment than was once thought [5–7]. It has innate resistance to many antibiotics and treatment is difficult [8, 9]. The prognosis for *B. cepacia*-positive CF patients is variable, ranging from asymptomatic carriage to a fatal necrotising pneumonia with septicaemic complications [10]. This latter progression is sometimes called ‘*B. cepacia syndrome*’ [11, 12].

Compelling evidence for person-to-person transmission of *B. cepacia* in CF patients has been established by molecular typing [13, 14] and the prevalence of certain highly transmissible strains has been described [15]. These include an epidemic strain, ET12, responsible for many *B. cepacia* infections in the UK and North America [15, 16]. This strain is highly transmissible, with even brief close contact appearing to permit passage between CF patients [15, 17, 18]. Segregative policies have reduced the incidence of *B. cepacia* in CF patients [16, 17], but reports describing the delayed detection of this organism highlight the importance of continued vigilance [1]. Distinguishing those strains that do and do not readily cause cross-infection has the potential to benefit *B. cepacia*-positive CF patients.

However, the role of epidemic strains is not universal. Polymerase chain reaction, ribotyping and pulsed-field gel electrophoresis (PFGE) identified eight distinct *B. cepacia* genotypes among isolates from the Danish CF population [19] and, in contrast to the UK and North
America, there was no evidence for nosocomial cross-infection, even though most CF patients regularly attend the same CF clinic [19].

Genomic analysis by PFGE is a powerful method for distinguishing strains in many species including B. cepacia [14, 20] but is expensive and demanding, and a simpler phenotypic characterisation might be useful in distinguishing between strains before a more detailed analysis is carried out. Analysis of outer-membrane protein (OMP) profiles by SDS-PAGE allows rapid, phenotypic characterisation and has been applied successfully to, e.g., Haemophilus influenzae [20]. In B. cepacia, the OMP profile is characterised either by a major protein with an apparent mol. wt of 39 kDa or a cluster of proteins in the 80–90-kDa size range [21]. Detailed analysis has shown that the high mol. wt cluster is a complex of subunits of 39 and 27 kDa, which resists denaturation by heat before electrophoretic analysis [22].

This study examined CF isolates of B. cepacia obtained from two sources (the UK and Denmark) by PFGE and OMP profiling and found that OMP profiles may provide a rapid means of making an initial distinction among isolates.

Materials and methods

Strains

Clinical isolates and laboratory strains of B. cepacia, B. gladioli and B. vietnamiensis were obtained from the sources indicated in Table 1. Each strain was stored in nutrient broth supplemented with glycerol 10% v/v at −70°C until use. Pseudomonas aeruginosa NCTC 6750, P aeruginosa ATCC 15692 and Stenotrophomonas maltophilia NCTC 10257 were included as controls. All strains were maintained on Mueller-Hinton Agar (Oxoid).

Table 1. Origin of strains and isolates used in this study

<table>
<thead>
<tr>
<th>Strain identifier</th>
<th>Source</th>
</tr>
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<tr>
<td>SBI; SJIV; SNVI; SII; SPIV; SPIV; 5QV; 5QV; 5QV; 5RIV; 5SI; 5QV</td>
<td>CF, Birmingham</td>
</tr>
<tr>
<td>5O; 5PI</td>
<td>Burn isolate, Birmingham</td>
</tr>
<tr>
<td>5RI; 5RII; 5RIII; C1948; J2315 (both epidemic ET12 isolates) J2552</td>
<td>Chronic granulomatous disease isolates, Birmingham</td>
</tr>
<tr>
<td>5SV; 5SVI; 5TI; 5TI; 5TI; 5TV</td>
<td>CF, Manchester</td>
</tr>
<tr>
<td>3843; 3925; 4325; 4326; 4949; 9275; 12794; 15836</td>
<td>CF, Edinburgh [15]</td>
</tr>
<tr>
<td>B. cepacia ATCC 17616</td>
<td>Botanical isolate, Edinburgh [7]</td>
</tr>
<tr>
<td>B. cepacia NCTC 10744</td>
<td>CF, Liverpool</td>
</tr>
<tr>
<td>B. cepacia NCTC 10661</td>
<td>CF, Copenhagen</td>
</tr>
<tr>
<td>B. gladioli ATCC 10248</td>
<td>Soil</td>
</tr>
<tr>
<td>B. gladioli ATCC 10854</td>
<td>Blood culture</td>
</tr>
<tr>
<td>B. gladioli ATCC 25417</td>
<td>Trinidad, forest soil</td>
</tr>
<tr>
<td>B. vietnamiensis LMG 6998</td>
<td>Gladiolus leaf</td>
</tr>
<tr>
<td>S. maltophilia NCTC 10257</td>
<td>Onion</td>
</tr>
<tr>
<td>P. aeruginosa NCTC 6750</td>
<td>Gladiolus leaf</td>
</tr>
<tr>
<td>P. aeruginosa ATCC 15692</td>
<td>Blood culture</td>
</tr>
<tr>
<td></td>
<td>Buccal cavity</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
</tr>
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<td></td>
<td>Wound infection</td>
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</tbody>
</table>

ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures (UK); LMG, University of Ghent Culture Collection, Belgium.

Preparation of high-mol.-wt genomic DNA

Fifty ml of Mueller-Hinton Broth were inoculated with a single colony from a 36-h culture grown at 37°C on Mueller-Hinton Agar. The liquid culture was then incubated for 48 h at 37°C with continuous shaking, before harvesting by centrifugation at 10 000 g for 10 min at 25°C. The cells were washed twice in sterile NaCl 0.9% solution and then resuspended in 1 ml of NET-100 (0.1 M NaCl, 0.1 M EDTA, pH 8.0, 0.1 M Tris-HCl, pH 8.0) at a density of 5 mg/ml (wet weight). This suspension was used to prepare agarose plugs for PFGE according to the method of Rainey et al. [23].

Restriction digestion of DNA and PFGE

Two restriction endonucleases were used: XbaI (which recognises the sequence 5'-TCTAGA) and DraI (5'-TTTAAAA). Portions (1 × 1 × 9 mm) of agarose plugs containing high mol.wt DNA were digested by the method of Rainey et al. [23] and samples were subjected to PFGE on a BioRad CHEF DRIII apparatus (CA, USA) with 0.5 × TBE (1 × TBE is 90 mM Tris-borate and 1 mM EDTA, pH 8.0) buffer in agarose (BioRad) 1.2%. The ramped pulse time was 7–60 s (XbaI digests) or 2–25 s (DraI) for 22 h at 6 V/cm with a 120° electrode angle. The gels were stained with ethidium bromide 0.5 mg/L for 30 min, de-stained in distilled water, and examined by means of a scanner (UVP Products, Cambridge). At least two independent separations of the restriction fragments were performed to confirm reproducibility.

Evaluation of pulsed-field gels

Band sizes were estimated with the Gelworks 1D software package (UVP Products) and the numerical method of Grothues and Tümmler [24] was used to assess statistically the relatedness of the strains. A computer-based spreadsheet (Microsoft Excel) was
used to assist in this process. Relatedness was
determined by comprehensive pair-wise comparison
of restriction fragment sizes within the sample group.
This permitted the calculation of the Dice coefficient
($S_D$) for each pair. The gel photographs were divided
into suitable intervals; $Xba$I-digested samples and
$Dra$I-digested samples were deemed to possess 28
and 40 intervals, respectively. Statistical significance
was then evaluated by the equation described by
Grothues and Tümmler [24], and the hierarchical
UPGMA method of analysis was used to group the
strains into clusters based on the $S_D$ values. The
computer-based statistical analysis software Unistat
(Version 4.0) was used for this step and to generate a
dendrogram of the data, working in unison with
Microsoft Excel.

**OMPs**

Mueller-Hinton broth cultures (1 L) were inoculated
with 50-ml amounts of overnight broth cultures and
incubated for 16 h with continuous shaking at 37°C.
The cells were then harvested by centrifugation at
10,000 $g$ for 10 min at 25°C, washed once in water,
then resuspended in 20 ml of water containing
deoxyribonuclease II 250 mg/L to reduce the viscosity
on sonication. The cells were then ruptured by
sonication (MSE Soniprep 150, MSE, Crawley, W.
Sussex) with a logarithmic probe of tip diameter 4 mm,
operating at maximum power for 30 cycles of 30 s
sonication, with intervening pauses of 30 s for cooling
on ice. Any remaining whole cells were removed by
centrifugation at 10,000 $g$, and the supernate was
adjusted to 35 ml with water. N-Lauroyl sarcosine
was added to 2% w/v, and the mixture was allowed to
stand for 5 min to solubilise the cytoplasmic mem-
branes. Centrifugation at 35,000 $g$ for 1 h at 4°C was
then used to deposit a pellet containing partially
purified outer membranes. This was resuspended in
35 ml of N-lauroyl sarcosine 2% w/v, left to stand for
5 min and re-centrifuged to yield a pellet containing
purified outer membranes. These were resuspended in
2 ml of water and stored at −20°C for analysis by SDS-
PAGE.

**SDS-PAGE of OMPs**

Electrophoresis was performed in acrylamide 12% w/v
gels prepared as described by Lugtenberg et al. [25].
Samples were denatured for 30 min at 100°C and
subjected to SDS-PAGE on a Mini-Protean apparatus
(BioRad). The gels subsequently were stained with
Coomassie Blue.

**Results**

**OMP profiles**

Fig. 1a shows the OMP profiles determined by SDS-
PAGE for several isolates. The profiles of *B. cepacia*,
*B. gladioli* and *B. vietnamiensis* each contained three
major proteins in the range 14–39 kDa. In *B. cepacia*,
two different protein profiles were observed when the
outer membranes were denatured at 100°C for 30 min.
The first, termed type A, contained two major proteins,
with mol. wt of 39 and 27 kDa, and a minor component
around 18 kDa. The second profile, termed type B,
contained an additional complex of high mol. wt
components in the region 80–90 kDa as well as the
39-, 27- and (minor) 18-kDa bands. All the clinical
isolates gave OMP patterns that could be grouped as
type A or type B: the UK isolates comprised 11 of type
A and 15 type B, whereas the Danish isolates belonged
exclusively to type A. The distinctive appearance of the
type A and B OMP profiles was obtained only after
denaturation for 30 min at 100°C. When less rigorous heating (80°C for 30 min) was used to denature the samples, all of the organisms produced type B profiles (Fig. 1b); whereas, after prolonged denaturation (100°C for 60 min) all profiles converted to type A (Fig. 1c).

Restriction digestion of the Burkholderia genome

Two rarely cutting restriction endonucleases, XbaI and DraI, were used to cleave the B. cepacia genome (Fig. 2), and yielded 28–30 bands. Other restriction endonucleases yielded too many or too few fragments for use in pairwise comparisons. Table 2 lists the $S_D$ calculated for representative strains, and a dendrogram showing the relatedness of the organisms is presented in Fig. 3. A single cluster is present above a distance of c. 0.58 Euclids, with subsequent subdivisions in evidence. The laboratory strains P. aeruginosa NCTC 6750 and ATCC 15692, S. maltophilia NCTC 10247 and B. gladioli ATCC 10248, ATCC 10854 and ATCC 25417 were discriminated from the majority of strains at distances of 0.58, 0.53 and 0.46 Euclids, respectively. Two clinical isolates from Manchester (SRII, 5RIII) did not group with the rest of the B. cepacia strains and, surprisingly, seemed to be most closely associated with S. maltophilia NCTC 10257, although they diverged from this laboratory strain at 0.45 Euclids. This is a higher level than that at which some separately designated species diverge from each other; e.g., B. cepacia and B. vietnamiensis (see below). A third clinical isolate from Manchester (SRI) was more closely associated with the remainder of the UK clinical strains.

Most of the clinical Burkholderia isolates divided into four main clusters at a level of 0.4 Euclids. These clusters are indicated as 1–4 in Fig. 3. Cluster 4 contained two isolates (J2315 and C1948) previously identified as the CF epidemic lineage ET12 [16]. This cluster appeared to divide into three subgroups (a, b and c in Fig. 3) at a level of 0.12 Euclids. Typically, representatives of these subgroups yielded distinctive patterns.
banding patterns when digested with XbaI or DraI (Fig. 2). These patterns were not found when other isolates were analysed. *B. cepacia* ATCC 10744, ATCC 17616 and NCTC 10661, and *B. vietnamiensis* LMG 6998 remained associated with these four clusters of clinical isolates until a distance of 0.41 Euclids, and diverged from each other at a distance of 0.31 Euclids. Thus, representatives of two distinct species – *B. cepacia* and *B. vietnamiensis* – were more closely related to each other than to some of the clinical isolates identified as *B. cepacia* [26]. Fig. 3 also indicates the OMP profile groups determined by SDS-PAGE. All the strains from Denmark belonged to type A and were clustered together in groups 1, 2 and 3 of the PFGE dendrogram (Fig. 3). The 15 UK strains with type B OMP profiles were found in PFGE clusters 3 and 4.

**Discussion**

Vandamme et al. [26] recently conducted a comprehensive analysis of presumed strains of *B. cepacia* associated with CF by analysis of whole-cell proteins, fatty acids and by a number of DNA hybridisation methods. This multi-faceted taxonomic approach identified at least five distinct genomic species, referred to as the *B. cepacia* complex. Further differential phenotypic tests are needed to determine the relative importance of members of this complex in CF, particularly members of genomovars I, III and IV [26].

The present study aimed to determine whether OMP profiles were a realistic predictor of genotype as determined by PFGE. Analysis of OMPs by SDS-PAGE following denaturation of outer membranes at 100°C for 30 min revealed two different types of profile among isolates from the UK and Denmark. These were distinguished by the presence (type B) or absence (type A) of a cluster of proteins in the region 80–90 kDa. All the isolates gave type B profiles when milder conditions were employed for denaturation (80°C for 30 min) and group A profiles when more vigorous and extended conditions were employed (100°C for 60 min). This temperature-dependent behaviour suggests that the 80–90 kDa proteins are more tightly associated together in the type B strains than in the type A strains and, consequently, were more resistant to denaturation by heat. Lipopolysaccharide (LPS) may play a role in the association of these proteins; previous studies have shown that the 80–90 kDa protein complex can be dissociated into its lower mol. wt components following electro-elution from the gels [27]. As electro-elution effectively removes LPS from the proteins [28] it is possible that, in the type B strains, the LPS is more tightly associated with these proteins than in the type A strains.

None of the eight CF isolates from Denmark was of type B, whereas a significant proportion (15 of 26) of the CF isolates from the UK, including the epidemic strain J2315, were of this type. The three *B. gladioli* and one *B. vietnamiensis* strains from culture collections all gave type A OMP profiles. It is interesting that these organisms, which are closely related to *B. cepacia*, share common features in their OMP profiles.

PFGE is a powerful analytical approach for distinguishing epidemiologically related isolates [29] and its application here confirmed the heterogeneity of *B.

**Table 2. Relatedness of representative strains, as based on PFGE analysis**

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>NCTC 10257</th>
<th>ATCC 10248</th>
<th>LMG 6998</th>
<th>NCTC 10744</th>
<th>4949</th>
<th>15856</th>
<th>5PI</th>
<th>5SV</th>
<th>5PIV</th>
<th>5JIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCTC 6750</td>
<td>0.44* (0.37)</td>
<td>0.41 (0.52)</td>
<td>0.35 (0.44)</td>
<td>0.39 (0.41)</td>
<td>0.45 (0.43)</td>
<td>0.41 (0.49)</td>
<td>0.41 (0.45)</td>
<td>0.42 (0.48)</td>
<td>0.39 (0.49)</td>
<td>0.39 (0.48)</td>
</tr>
<tr>
<td>NCTC 10257</td>
<td>0.50 (0.53)</td>
<td>0.47 (0.44)</td>
<td>0.36 (0.42)</td>
<td>0.34 (0.44)</td>
<td>0.40 (0.52)</td>
<td>0.44 (0.46)</td>
<td>0.40 (0.47)</td>
<td>0.43 (0.48)</td>
<td>0.41 (0.48)</td>
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<tr>
<td>ATCC 10248</td>
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<td>0.49 (0.59)</td>
<td>0.50 (0.63)</td>
<td>0.45 (0.74)</td>
<td>0.53 (0.65)</td>
<td>0.57 (0.69)</td>
<td>0.57 (0.71)</td>
<td>0.60 (0.71)</td>
<td>0.54 (0.70)</td>
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<tr>
<td>LMG 6998</td>
<td>0.69 (0.49)</td>
<td>0.52 (0.53)</td>
<td>0.63 (0.60)</td>
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<td>0.60 (0.51)</td>
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<td>0.57 (0.55)</td>
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<tr>
<td>4949</td>
<td>0.86 (0.60)</td>
<td>0.61 (0.61)</td>
<td>0.60 (0.61)</td>
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<td>15856</td>
<td>0.51 (0.64)</td>
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<tr>
<td>5PI</td>
<td>0.86 (0.65)</td>
<td>0.85 (0.66)</td>
<td>0.87 (0.68)</td>
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<td>5SV</td>
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<td>0.85 (0.66)</td>
<td>0.87 (0.68)</td>
<td>0.86 (0.68)</td>
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<td>5PIV</td>
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<td>0.87 (0.68)</td>
<td>0.86 (0.68)</td>
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</table>

*SD* between selected pairwise comparisons within the sample (top number in each cell) compared to the lower limit of the 99% confidence level (in parentheses). If the *SD* is lower than the confidence limit, then the two strains are significantly dissimilar. Values in bold type represent significant dissimilarities.
cepacia isolates from CF patients described by Vandamme et al. [26]. Four clusters were found, compared with at least five distinct genomic species reported by Vandamme et al. [26], who based their conclusions on a combination of phenotypic and DNA hybridisation methods. The Danish isolates were confined to clusters 1, 2 and 3 and shared common type A OMP profiles. All the isolates in clusters 1-4 were less similar to reference isolates of B. cepacia than to an isolate of the closely related species, namely B. vietnamiensis. B. cepacia and B. vietnamiensis were placed at a distance of c. 0.36 Euclids from each other (Fig. 3). Recent studies by Gaur and Wilkinson [30] have identified the same polysaccharide structure in the LPS of the O4 serotype of B. cepacia, and a strain of B. vietnamiensis, which gives further evidence of their close relationship. Other isolates, considered to be B. cepacia in the clinical setting, diverged from these two species at lower levels of similarity (i.e., > 0.36 Euclids). This result supports a previous review [16] questioning exactly what constitutes ‘B. cepacia’.

The lower ends of the 99% confidence limits between certain strains in cluster 4 and reference strains are shown in Table 2, together with the actual $S_D$. The

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**Fig. 3.** Dendrogram showing relatedness of the strains, created via cluster analysis of the $S_D$ by the UPGMA method.
strains included were selected for their positions within the dendogram (Fig. 3). The lower end of the confidence range is critical here, as this study is concerned primarily with differences between strains, and not with their similarity: if the calculated $S_p$ are lower than the confidence value, it is certain that the organisms are significantly different. These confidence values must be viewed with some caution. The confidence equation [24] is heavily dependent upon $I_K$, the interval factor, so small variations in $I_K$ have a fairly large impact on the confidence value. Moreover, $I_K$ is the most subjective parameter in the confidence equation. For these reasons, the results of the confidence equation must be interpreted carefully.

The values in bold type in Table 2 indicate the positions where statistical analysis returned a result of significant dissimilarity. The isolates falling within the cluster (PFGE cluster 4) containing representatives of the CF epidemic ET12 lineage were significantly dissimilar to all isolates except B. viennensis LMG 6998 and B. cepacia NCTC 10744 and, in the case of isolate 5JIV only, isolate 5PI.

Previous PFGE studies by Pitt et al. [31] demonstrated four distinct patterns among ET12 isolates, differing by three or fewer bands, one of which was exclusively associated with strains of restricted metabolic capacity. From direct observation, cluster 4 appears to split into three subgroups, designated a, b and c (Fig. 3), with the ET12 isolates falling in cluster 4a. From our previous research, it appears that subgroup clusters differ in metabolic capabilities [32]. Thus cluster 4b contains several strains (5PIV, 5PV1, 5RIV and 5TI) that appear to be auxotrophs and others with limited metabolic capabilities, such as 5QIV [32]. The high prevalence of auxotrophic strains in the CF lung is reasonably explained by Barth and Pitt [33] as being caused by the abundance of nutrients arising from pulmonary damage. More generally, however, a reliance on growth factors and a narrow metabolic capability is not commonly considered a property of B. cepacia sensu stricto.

Based on these data, it appears that the species definition of B. cepacia has broadened since its original description by Burkholder in 1950 [34] to include several closely related potential species. Further evidence to support this conclusion is provided by Yohalem and Lorbeer [35, 36] and the presence of novel species among isolates currently considered as B. cepacia – but fitting this species description poorly – has also been suggested by others [16, 26, 37].

The extremes of clinical outcome observed after B. cepacia infection in CF may well be rationalised by closer examination of the identity of the infecting organism; however, clinical outcome may not always be predictable even in patients colonised with the same strain. Interactions between host and pathogen are clearly important as most CF individuals, including those infected with the notorious ET12 lineage and other strains of genomovar III, do not succumb to ‘B. cepacia syndrome’ [15, 16]. Nevertheless, more detailed phenotypic and genomic analysis may reveal a subpopulation of Burkholderia with a feature promoting adverse clinical outcomes.

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References


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