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Genomic diversity and security profile of *Corynebacterium pseudodiphtheriticum* species

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INTRODUCTION

Bacteria belonging to the genus *Corynebacterium* have usually been associated with infectious diseases. *C. diphtheriae* and *C. ulcerans* produce toxins that can induce upper respiratory tract illness characterized by sore throat, fever, and nasal discharge. However, not all the species of this genus are pathogenic. *C. pseudodiphtheriticum* is usually found as a member of the normal microbiota of the upper respiratory tract and some studies have indicated that these bacteria exert beneficial effects for the host. In this regard, we have shown that the strain *C. pseudodiphtheriticum* 090104, isolated from the human nasal mucosa, is able to beneficially modulate immune responses in the respiratory tract, improving the resistance to pathogens such as *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and respiratory syncytial virus [1-3]. Considering the positive effects of this species, we aimed to carry out a comparative genomic study of *C. pseudodiphtheriticum* strains to characterize their security profile in terms of virulence and antibiotic resistance genes.

MATERIAL AND METHODS

Genome Selection: Genomic sequence data for *C. pseudodiphtheriticum* strains were obtained from the National Center for Biotechnology Information (NCBI) database. A total of 29 genomes (including the strain 090104) were selected based on their completeness and quality control.

Average Nucleotide Identity (ANI) Analysis: ANIm was used as a parameter for our comparative genomic analysis. ANIm calculation of the 29 strains was carried out using the JspeciesWS online server [4]. From these data, an ANI heatmap was constructed using the heatmap.2 function of the gplots R package (version 3.1.3).

Genome annotation and pangenome analysis: The 29 genomes of *C. pseudodiphtheriticum* were re-annotated using the Prokaryotic Genome Annotation System (Prokka), v1.14.5 [5]. Functional annotation was performed through the Rapid Annotations Subsystems Technology (RAST)[6]. The GFF3 files generated by Prokka were used for the analysis utilizing Roary v3.11.2 default settings [7].

Virulence and antibiotic resistance genes: The presence and absence of the genes was study using the blast algorithm. The heatmaps were constructed as specified above.

Minimum inhibitory concentration (MIC) determination: Antimicrobial susceptibility tests were performed using the broth microdilution method as recommended by the CLSI for infrequently isolated or fastidious bacteria (M45-A, CLSI-2016). The antimicrobial agents were penicillin (PEN), cefotaxime (CTX), ceftriaxone (CRO), meropenem (MER), vancomycin (VAN), gentamicin (GEN), ciprofloxacin (CIP), erythromicin (ERY), tetracycline (TET). MIC results were interpreted following CLSI guidelines (M45-A, CLSI-2016).

RESULTS

The ANIm analysis of the 29 selected strains revealed identity percentages greater than 95% (**Figure 1**). The strains were categorized into two distinct groups. The strain 090104 was grouped together with the strains CPD, CP10, MSK037, MSK090, MSK092, MSK305, and DSM44287; whereas the remaining 21 strains formed a second cluster. Pangenome analysis revealed a pangenome composed of 5050 genes (**Figure 2A**), which appears to remain open (**Figure 2B**). Of these genes, 1471 correspond to the core-genome, 1528 to softcore, 1061 to shell-genome and 2461 to the cloud-genome (**Figure 2A**). In accordance with the phylogenetic data obtained by the ANI analysis, strain 090104 was grouped in a cluster composed of the same strains in the pangenomic analysis.

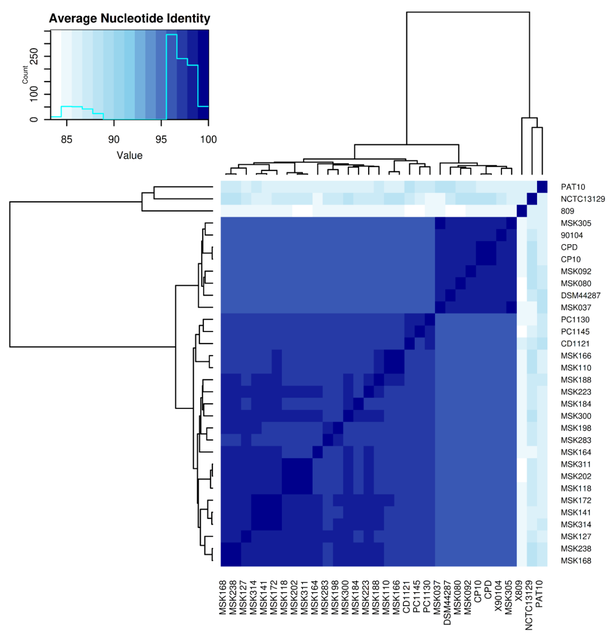

Figure 1. Heatmap of average nucleotide identity (ANIm) values for *C. pseudodiphtheriticum* strains.

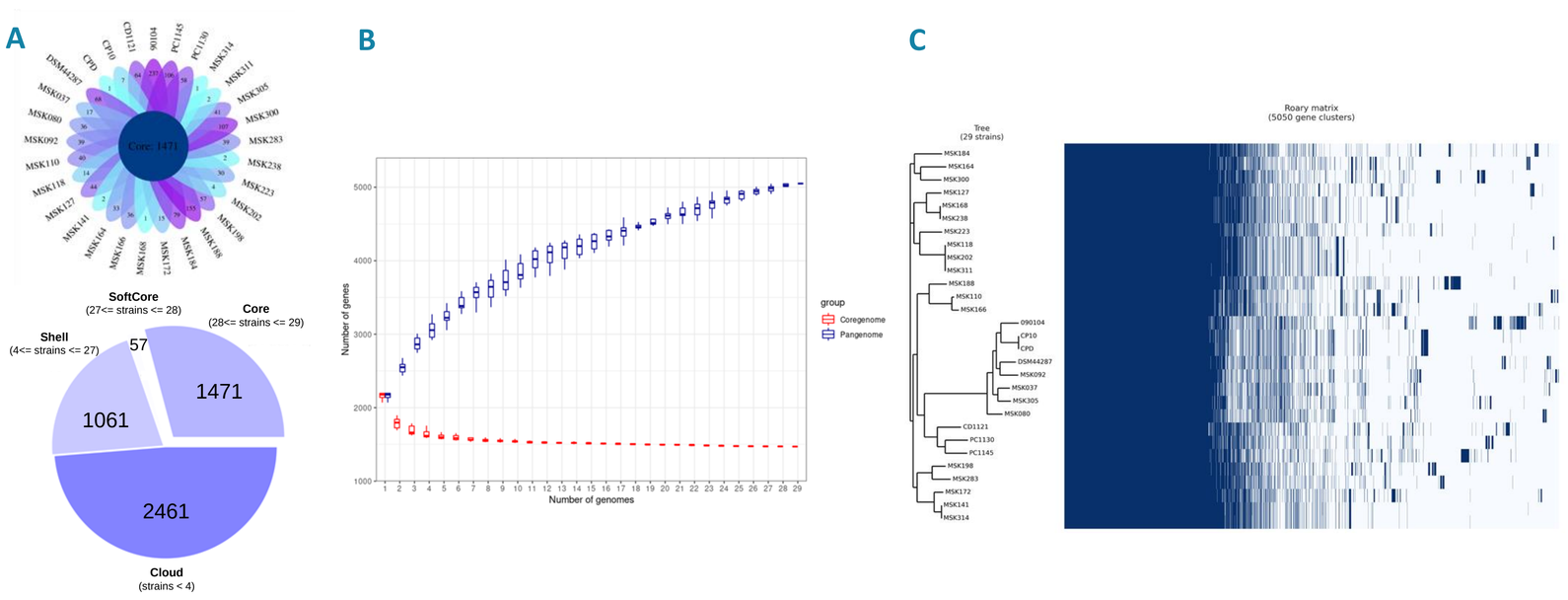

Figure 2. Pangenome analysis with Roary. (**A**) The core and unique genes are shown in the flower plot diagram and the number of core, shell and cloud genes are shown in the pie chart. (**B**) The evolution of the pangenome and coregenome as new genomes are added is represented. (**C**) Dendrogram constructed from the 5050 genes of the pangenome.

Virulence and antibiotic resistance genes were analyzed. The presence of the virulence genes *dip0733*, *dtxr*, and genes linked to secretion systems (*t2sf* and *sec* genes) was observed in all the strains (**Figure 3**). The virulence gene *hmuv* was found to be present in 27 of 29 strains (absent in MSK037 and MSK092).

The search for resistance genes revealed that all the strains studied have several erythromycin resistance genes (*rsmE*, *rsmG*, *rsmH*, *rsmI*), sulfonamide resistance genes (*folp1*, *folp2*) and at least one beta-lactamase (**Figure 4**). The MSK184 strain presented the greatest variety and number of antibiotic resistance genes. Considering the results, the study of the sensitivity of *C. pseudodiphtheriticum* 090104 to different routine antibiotics was performed, according to CLSI guideline (**Table 1**). *C. pseudodiphtheriticum* 090104 presented resistance to beta-lactam antibiotics (PEN, CTX, CRO) and macrolides such as ERY.

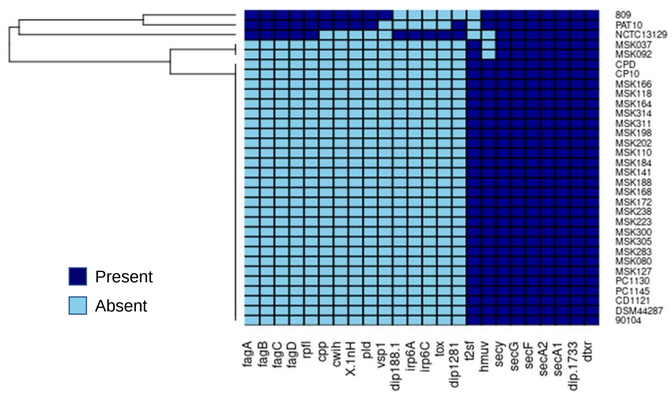

Figure 3. Heatmap of virulence genes in *C. pseudodiphtheriticum* strains.

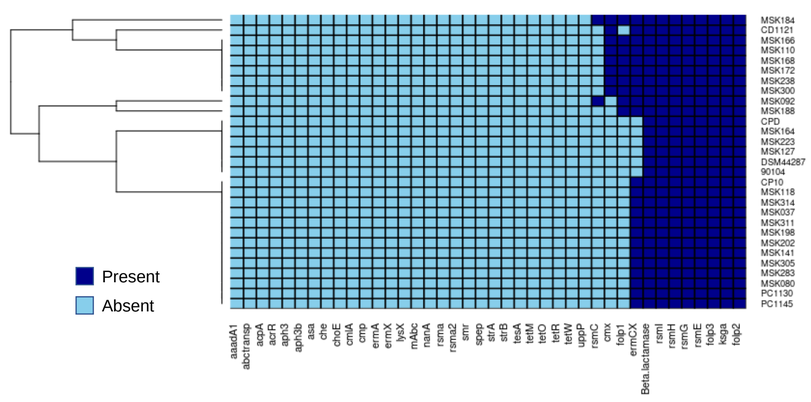

Figure 4. Heatmap of resistance genes in *C. pseudodiphtheriticum* strains.

Table 1. Antibiotic resistance of *C. pseudodiphtheriticum* 090104 according to CLSI guideline

Antimicrobial Agent	MIC (µg/mL) Interpretive Criteria		MIC (µg/mL) Cp090104	Results
	S	R		
Penicillin	≤ 0,12	≥ 4	≥ 32	R
Cefotaxime	≤ 1	≥ 4	≥ 32	R
Ceftriaxone	≤ 1	≥ 4	≥ 32	R
Meropenem	≤ 0,25	≥ 1	≤ 0,5	S
Vancomycin	≤ 2	> 4	≤ 0,05	S
Gentamicin	≤ 4	≥ 16	≤ 0,5	S
Erythromycin	≤ 0,5	≥ 2	4	R
Ciprofloxacin	≤ 1	≥ 4	≤ 0,125	S
Tetracycline	≤ 4	≥ 16	≤ 0,5	S

Breakpoints for the different antibiotic are shown. MIC: Minimum inhibiti concentration. R: resistant, S: susceptible.

CONCLUSIONS

C. pseudodiphtheriticum strains have low potential to generate infections due to the low number of virulence genes. However, although the risk for health is small, it is advisable to perform detailed *in vitro* and *in vivo* studies of the strains intended to be used in probiotic formulations, to ensure its complete safety. Furthermore, considering the presence of resistance genes for beta-lactams, sulfonamides and erythromycin, the use of the dead bacteria would be advisable.

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