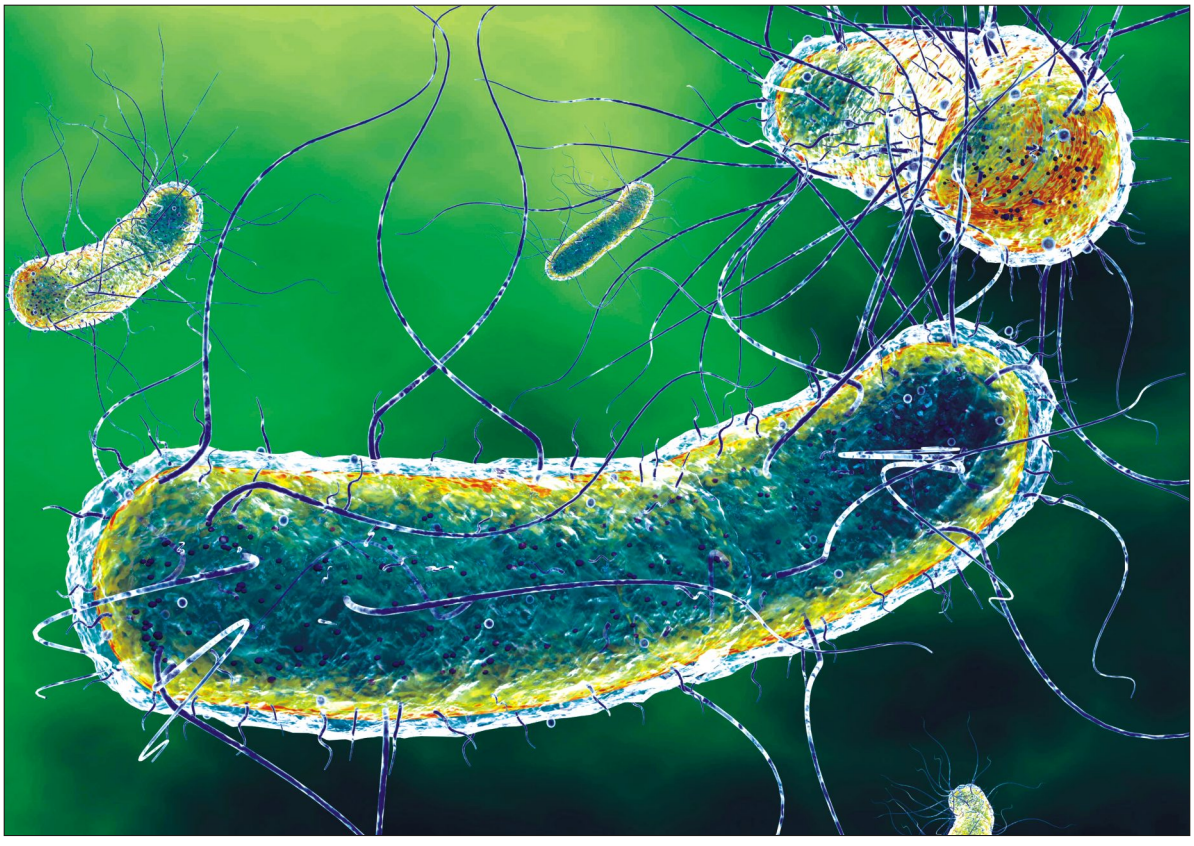

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Research Article
Cloning of an Internal Fragment of *pimA* Gene Coding Glycosyl-transferase of *Corynebacterium glutamicum*

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Abstract

Background and Objectives: Mycolyl-transferases are a clan of proteins that are especially present in the CMN genera (*Corynebacterium*, *Mycobacterium* and *Nocardia*), mycolyl-transferases are responsible for cell wall components synthesis. The mycobacteria and corynebacteria envelopes share some gross structural features and similar cell wall architecture. The aim of the present work is to identify *C. glutamicum* genes encoding for glycosyl-transferases enzymes activity. **Materials and Methods:** *In silico* search for "glycosyl transferases" that were common both to *M. tuberculosis* and *Corynebacterium difteriae* revealed the presence of PimA-like sequences in the actinomycete *Streptomyces coelicolor* and in the extremophile archeons *Pyrococcus horikoshii*, *Aeropyrum pernix* and *Pyrococcus abyssi*. **Results:** Highly conserved regions obtained permitted the design of mixtures of oligonucleotides pairs intended to PCR amplification of a *pim*A gene fragment. The integration of the internal *pim*A gene fragment at the bacterial genome was done by a single homologous recombination event at the identical wild-type *pim*A gene of *C. glutamicum* Or2262. The *pim*A gene (belonging to the locus *pgs*A-*htr*B-*pim*A) and encoding for glycosyl-transferase enzyme activity of the species *C. glutamicum* ATCC13032 and *C. glutamicum* sp. 2262 (reclassified as *C. glutamicum* Or2262) was successfully cloned. **Conclusion:** A comparison of the transformability of *C. glutamicum* Or2262 and f. *C. glutamicum* ATCC13032 RES167 revealed 18.0 times difference in the ratio of transformability, which suggested that it is attributed to the difference in the efficiency of plasmid-host recombination rather than the efficiency of diffusion of the plasmid through the bacterial envelope.

Key words: Cloning, *Corynebacterium glutamicum*, glycosyl-transferase, mutants, *pimA*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Around 33% of the total populace is infected with *Mycobacterium tuberculosis*, the etiological agent of tuberculosis (TB) which remains the leading reason for mortality from a single infectious organism. The steadfastness of this pathogen is linked with its unmistakable cell divider structure. *Mycobacterium tuberculosis* has an odd lipid-rich cell wall including an ample collection of antigens, providing a protective hydrophobic leak-proof barrier against antibiotic drugs. This sophisticated and unusual structure is important for the growth, viability and infectious ability of this micro-organism thus, attracting attention as a goal for developing drugs and vaccines[1,2].

A good deal of the available information of the functional features of the *Corynebacterium* envelope is derived from studies of the secretion of metabolites of economic importance by non-pathogenic strains from the *Corynebacterium glutamicum* group. Most of the evidence on the chemical and structural features, the *Corynebacterum* genus come from pathogenic strains studied in the context of the mycobacterial pathogenesis and from extrapolations from the important amount of knowledge on the mycobacterial envelope[3-5]. A more specific view of the corynebacterial envelope has been proposed in an experimental article that reviews the current status of corynebacterial structural research[6].

Although, they are Gram-positive bacteria, corynebacteria and closely related micro-organisms share with enteric Gram-negative bacteria, the property having a strong permeability barrier other than the plasma membrane. In Gram-negative micro-organisms this additional barrier is accounted for by a complex outer layer of structure and composition different to other bacterial plasma membrane. Unique features of this structure are the presence of inositol, only found in eukaryotic cells and some archaebacteria and the presence of a unique type of lipids, the mycolates and corynomycolates. An updated perusal of the published literature agreed that the mycobacteria and corynebacteria envelopes share some gross structural features and similar cell wall architecture[7].

Arabinogalactan (AG) and lipoarabinomannan (LAM) which are arabinan-containing polysaccharides are key components of the cell wall of Corynebacterineae, which comprise corynebacteria, norcardia and mycobacteria[8].

However, fundamental differences between these two genus are found in the composition of these features. Analysis by quantitative sugar and glycosyl linkage showed that *C. glutamicum* have a tiny version of LAM, called Cg-LAM[9].

Mycolyl-transferases are a clan of proteins that are especially present in the CMN genera (*Corynebacterium, Mycobacterium* and *Nocardia*), mycolyl-transferases are responsible for cell wall components synthesis[10]. The cell walls of the members of CMN genera organisms are composed of linked peptidoglycan and polysaccharide-mycolate complex and are attributed by the existence of mycolic acid on their surface[11].

The micro-organisms belonging to CMN genera are gathered together as a group on the ground of some aspects including complicated cell wall components, type and presence of mycolic acids, adjuvant activity, presence of cord factor, sulfo-lipids, iron-chelating compounds, polyphosphate and serological cross-reactivity.

Conceptually, the mycobacterial and corynebacterial envelopes are composed of two coats of complex structure: an outer coat and an inner coat. Indeed, a complete cell-wall lipid bilayer is observed even in a *C. glutamicum* mutant whose cAGM content is further reduced to half the wild-type value. The current models of the corynebacterial envelope structure of the cell-wall envelope[6,12] proposed that the cell-wall lipid bilayer is mostly formed essentially of small free lipids, predicting a 2-3 nm for the thickness of the bilayer-associated electron-transparent layer. Thus, the cell-wall lipid bilayer cannot account for the observed thickness of the corynebacterial Electro Transparent Layer (ETL) structure. This observation also reopens earlier questions raised about the nature of the ETL in mycobacteria[4,6].

Although, many studies concerned with physiological and gene cloning aspects of *Escherichia coli*[13] and *Corynebacterium* were executed[14,15] whether expressed in *Escherichia coli*[16,17] or *C. glutamicum*[5], but genes coding of glycosyl-transferases of *C. glutamicum* were not been among them.

As *C. glutamicum* and *M. tuberculosis* share a similar cell wall architecture, enabling the utilization of *C. glutamicum* as a model for the identification and study of, otherwise; essential, mycobacterial genes involved in lipomannan (LM) and lipoarabinomannan (LAM) biosynthesis[7]. Understanding the genes coding of glycosyl-transferases of *C. glutamicum* would help among other things to develop antibiotics against *M. tuberculosis*. The aim of the present work is to identify *C. glutamicum* genes encoding for glycosyl-transferases enzymes activity.

MATERIALS AND METHODS

Study area: This study was conducted from March, 2000 to December, 2003 at Institut de Génétique et Microbiologie (IGM), Université Paris XI-Sud, France and from March, 2019-2020 at Colleges of Applied Medical Sciences, Medical Rehabilitation Sciences, Pharmacy at Taibah University, Saudi Arabia.

Bacterial strains and growth conditions: Bacterial strains and vectors used throughout the work are listed in Table 1 and 2. Growing of *Corynebacterium* strains was carried out aerobically (250 rpm) at 34°C, either in Brain Heart Infusion (BHI; Difco) (Fisher Scientific, USA) rich medium or in MCGC minimal medium as described by Von der Osten *et al.*[18], except that citrate (used as a chelating agent) was replaced by deferoxamine. Following the growth of *Corynebacterium* strains was done by measuring the optical density at 570 nm (OD_{570}) in a DU 7400 Beckman spectrophotometer (Irvine, USA). Kanamycin and chloramphenicol were added to medium when needed at concentrations of 25 and 15 μg mL^{-1}, respectively. Growing of *Escherichia coli* strains was carried out aerobically at 37°C in Luria Bertani (LB) medium (Difco) (Fisher Scientific, USA) . Following the growth of *E. coli* strains was done by measuring the optical density at 600 nm (OD_{600}). Ampicillin, kanamycin and chloramphenicol were added to medium when needed at concentrations of 100, 25 and 30 μg mL^{-1}, respectively. All chemicals used were purchased from Sigma (Sigma-Aldrich, Missouri, USA).

DNA manipulations: Basic molecular biology methods used were done as described by Sambrook *et al.*[23]. Enzymes were purchased from Promega (Madison, USA), Biolab (Middlebury, USA) and Boehringer Mannheim (Rotkreuz, Switzerland). Extraction of plasmidic DNA was done by using the Wizard kit from Promega (Madison, USA). Isolation of DNA fragments

Table 1: Bacterial strains used in this study

Strains	Principle characteristics	References
Corynebacterium melassecola		
ATCC17965	Wild-type strain	ATCC
Corynebacterium glutamicum		
ATCC13032	Wild-type strain	ATCC
RES167	Negative restriction derivative of the strain ATCC13032	Via Kalinowski
ORSAN2262	Wild-type strain	ORSAN
2262.1	ORSAN2262 *pimA* :: pSG1 (Kan^r)	This study
2262.2	ORSAN2262 *pimA* :: pSG1 (Kan^r)	This study
167.1	RES167 *pimA* :: pSG1 (Kan^r)	This study
Escherichia coli		
TOP 10	F^-Δ(*mrr-hsdRMS-mcrBC*)ϕ80*dlacZ*ΔM15Δ*lacX74 recA1 deoRaraD139*Δ(*ara-leu*)7697*gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
JM110	F' traD36 $lacI^q$Δ(lacZ)M15 $proA^+B^+$/rpsL (Str^r) thr leu thi lacY galK galT ara fhuA dam dcm supE44 Δ(lac-proAB)	Yanisch-Perron *et al.*[19]
DH5α	F^-, Δ(*lac-argF*)U169, *recA1*, *endA1*, *hsdR17*(r_k-m_k^+), *supE44*, *gyrA*1, *relA1*, *deoR*, *thi-1* (ϕ80*dlacZ*ΔM15)	Woodcock *et al.*[20]

ATCC: American type culture collection, Rockville, USA

Table 2: Plasmids used or constructed in this study

Plasmids	Principle characteristics	Origin or reference
pCR 2.1-TOPO	Cloning vector for PCR products, origin of replication pUC and Amp^r Kan^r carrying the fragment *LacZ*∝	Invitrogen
pCGL0243	Shuttle Plasmid, pACYC184 origin for *E. coli*/pBL1 origin for *Corynebacterium*, Kan^r	Reyes *et al.*[21]
pSG1	Integrative vector derived from pCR 2.1-TOPO carrying the internal *pimA* fragment of 550 pb of *C. glutamicum* ORSAN2262	This study
pCGL0609	Acc No. AF092931	
pBAD18	Acc No. X81838	Guzman *et al.*[22]
pBAD28	Derived from pBAD18:Cm^r containing origin of replication pACYC1 of *E. coli*	Guzman *et al.*[22]
pCGL3155	Acc. No. Ul2390.pSport-1 at *Nar*I [552], the fragment 138-1630 *Cla*I of pCGL0609	
pCGL3177	Derived from pBAD28 and pSG4 pSG4: fragment *Asp718* - *Hind*III of 4,41 kbp in the deletion of 39 bp *Asp7*l8 - *Hind*III of plasmid pBAD28	This study
pCGL3180	Derived from pSG2 and pCGL3177 pCGL 3177: fragment *Hind*III- *BslB*I of 7,83 kbp ligated with the plasmid pSG2: fragment *Hind*III-*BstB*I of 670 bp. Cm', Km', Amp' containing the three genes (*pgsA*, *htrB* and *pimA*)	This study
pCGL3181	pCGL3180 cut with *Apa*LI-*Bsp*I ligated to fragment *Not* I and *Apa*LI of pCGL3155 (2466 bp) [231 to 2697) containing the gene *aphA-3*. Production of internal deletion at ORF *pgsA*	This study
pCGL3182	Derived from pCGL3180 and pCGL1315: pCGL3180: *Acl*I-*Cla*I [845-2205) of pCGLI1315	This study
pCGLI1315	pCGL0243 (fragment *Sac*I fragment) in pBluescript SK II (+) digested by *Sac*I (+) *Sac*I	Reyes *et al.*[21]
pBluescript SK II (+)	Acc. No. X52325: pBluescript SK(+)	

Ampr: Resistance for the ampicilline, Kanr: Resistance for the kanamycine

from agarose gels was done by using the Jetsorb kit (Genomed, Florida, USA). Extraction of chromosomal DNA of *C. glutamicum* was done by the method described by Ausubel *et al*.[24]. Transformation of *Corynebacterium* strains was done by electroporation[25]. Southern blotting technique was used for checking the integration of the introduced DNA into the chromosome DNA of *Corynebacterium*[26]. The DNA probes used were prepared and labeled non-radioactively using the DIG DNA Labeling and Detection kit (Roche, Basel, Switzerland). The colonies held for more studies were purified on selective solid medium. Storing of strains was done by picking up 1 mL of liquid medium of stationary-phase cultures inoculated with a single colony; mixing it with 1 mL of 80% glycerol, storing at -20°C.

Determination and analysis of the nucleotidic sequences: Nucleic acid sequences were determined by ESGS society. The method used for nucleic acid sequences determination was of Sanger *et al*.[27]. Analysis of the nucleotidic sequences was carried out using the software GeneJockey (Sigma-Aldrich, Missouri, USA), DNA Strider (LabStrider), Blast (NCBI) and CLUSTAL W (Clustal Omega).

PCR amplification of a *pim*A gene fragment: A pair of oligo-nucleotide mixtures (MT1: [GACGTBCTNCAYACGARCC] and MT2: [CCATGGCYTCKACSAGVACGATGCC] was used for PCR amplification of the chromosomal DNA of the *C. glutamicum* sp. 2262 strain. The reaction mixture for PCR amplification contained 2.5 U of thermostable DNA polymerase (Ampli*Taq*Gold, Perkin-Elmer), 30 ng of genomic DNA, 0.2 mM each deoxynucleotide triphosphates (Promega) (Madison, USA), 2 mM $MgCl_2$, 1×Ampli *Taq* Buffer (Thermo Fisher Scientific, USA) in a final volume of 50 µL and 0.5 µM each of both primers. The first cycle (10 min at 94°C) followed by 35 identical cycles (1 min at 94°C, 1 min at 50°C and 1 min at 72°C) then followed by a final elongation cycle (5 min at 72°C).

RESULTS AND DISCUSSION

Identification of target glycosyl transferases: The NCBI and Sanger Institute databases were searched for "glycosyl transferases" that were common both to *Mycobacterium tuberculosis* and *Corynebacterium difteriae*. This search identified a conspicuous protein, a cellobiosyl-diphosphoprenyl alpha-mannosyl transferase hereafter named "PimA" (*pimA*). PimA-like sequences are represented in several instances in the taxonomically related *M. tuberculosis*, *Mycobacterium leprae* and *C. diphtheriae*. Similar sequence to PimA was also present in the actinomycete *Streptomyces coelicolor* and in the extremophile archeons *Pyrococcus horikoshii, Aeropyrum pernix* and *Pyrococcus abyssi*. In the same trend, Berg *et al*.[28] showed that glycosyltransferases (Gts) of *M. tuberculosis* have orthologs in prokaryotes and eukaryotes.

Amplification of an internal *pimA* ORF fragment by PCR: Alignments in Sanger Institute of the presumed PimA proteins of *C. diphteriae* (contig 358- 373623:374684), *M. tuberculosis* (A70571) and *M. leprae* (CAB09632.1) showed highly conserved regions which permitted the design of mixtures of oligo-nucleotides pairs intended to PCR amplification of a *pimA* gene fragment devoid of the sequences needed to encode the PimA carboxy-terminal and amino-terminal regions. The chosen pair of oligonucleotide mixtures can be schematized as MT1 (GACGTBCTNCAYACGARCC) and MT2 (CCATGGCYTCKACSAGVACGATGCC) with respect of the codon preferences observed in *Corynebacterium glutamicum* and related species[29]. In theory, the oligonucleotides MT1 and MT2 could amplify a DNA band of about 550 bp located in the central portion of the *pimA* gene which exclude the amino-terminal and carboxy-terminal ends of the gene ORF. An amplified DNA band of the expected size (0.55 kpb) was obtained from the strain *Corynebacterium* sp. 2262[30], which will be hereafter referred to as *"C. glutamicum* Or2262*"* in basis of its high DNA sequence identity to *C. glutamicum* ATCC13032 and in the presence on it of corynebacterial LAM. This fragment was cloned into PCR 2.1-TOPO vector (Invitrogen®, California, USA). Transformants of the expected structure were identified by sequencing the insert ends using the oligonucleotides primers F-20 (M13 Forward-primer; GTAAAACGACGGCCAGT) and REV (M13 Reverse-primer; CAGGAAACAGCTATGACC). One of such plasmids pSG1 contained an insert highly homologous to the *pimA* genes of the mycobacteria and of *C. diphteriae.* Figure 1 showed amplification and cloning of a glycosyl transferase fragment *C. glutamicum* Or2262. This suggested that pSG1 insert correspond to a fragment of a *Corynebacterium* sp. 2262 gene that belongs to the *pimA* family.

Cloning of the internal fragment of *pim*A gene in *C. glutamicum* Or2262: The plasmid pSG1 carries the gene *aph*II, encoding for an aminoglycoside-phosphotransferase which determined resistance to kanamycin (KmR phenotype). Since, pSG1 is not replicative in *C. glutamicum*, most Km^r transformants should arose by homologous recombination

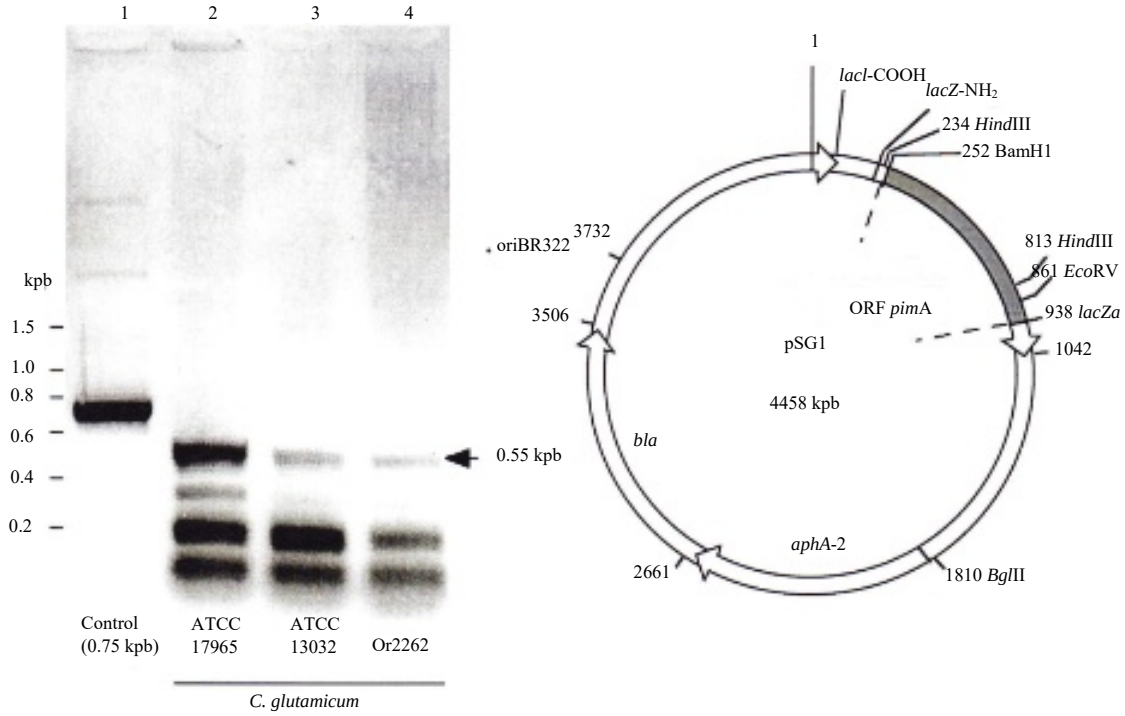

Fig. 1: Amplification and cloning of a glycosyl transferase fragment *Corynebacterium glutamicum* Or2262

with the cognate host chromosomal region. If plasmid pSG1 contains as expected, an internal *pimA* gene fragment, integration at the bacterial genome by a single homologous recombination event at the identical wild-type *pimA* gene of *C. glutamicum* Or2262 should lead to host *pimA* inactivation. Figure 2 showed interruption of *pim*A gene of fragment *C. glutamicum*. It refer hereafter to the *pimA* mutant allele further studied as *pimA1*. It also isolated insertions of pSG1 in *C. glutamicum* ATCC13032 RES167, one of is denominated *pimA2*. The transformability of *C. glutamicum* Or2262 (Table 3) is 18.0 times lower than that of *C. glutamicum* ATCC13032 RES167 as evaluated with the highly transformable plasmid pCGL0243, replicative in *C. glutamicum*. The transformability of *C. glutamicum* Or2262 with the integrative plasmid pSG1 is 71.5 times lower than that of the strain *C. glutamicum* ATCC13032 RES167, despite the fact that the sequence homology that determines host recombinational integration of pSG1 is perfect in the case of the former, which suggested that the limiting step in the efficiency of integrative transformation of *C. glutamicum* Or2262 is not diffusion of the plasmid through the bacterial envelope, but an efficiency of plasmid-host recombination lower than that of *C. glutamicum* ATCC13032 RES167. In corynebacteria, very high voltage(up to 40 kV cm^{-1}) electro-transformation has been achieved for intact C. *glutamicum* cells, though temperature and pressure effects limited the transformant yield to 3×10^3/µg of DNA[31]. Bonamy *et al.*[25] reported successful and efficient electro-transformation of plasmid DNA into intact cells of nine corynebacteria strains belonging to *Brevibacterium lactofermentum*, *Brevibacteriuum flavum*, *C. glutamicum* and *Corynebacterium melassecola*. In optimal conditions, more than 10^7 transformants per/µg of DNA could be obtained.

Accepted FMTR (Average frequency of replicative transformation) values were 3.84×10^{-5} for *C. glutamicum* ATCC13032 RES167 and 4.6×10^{-5} for *C. glutamicum* Or2262.

Isolation of the affected mutant in the *pim*A gene of *C. glutamicum*: The partial sequence of the plasmid containing the fragment of pSG1 and its insertion site directly indicated that the insertion of pSG1 defined mutations in the *pim*A gene. The genomic DNA of three pSG1 insertions in *C. glutamicum* Or2262 were digested with *Bam*HI, *EcoR*V and *Hind*II and then were subjected to hybridization analysis with pSG1 probe. Analysis results was found to be consistent with integration by homologous recombination of plasmid pSG1 in the host *pim*A gene. The integrated of *pimA* allele and the prediction of hybridization bands are shown in Fig. 3a-b, respectively. Southern hybridization of pimA mutants DNA of

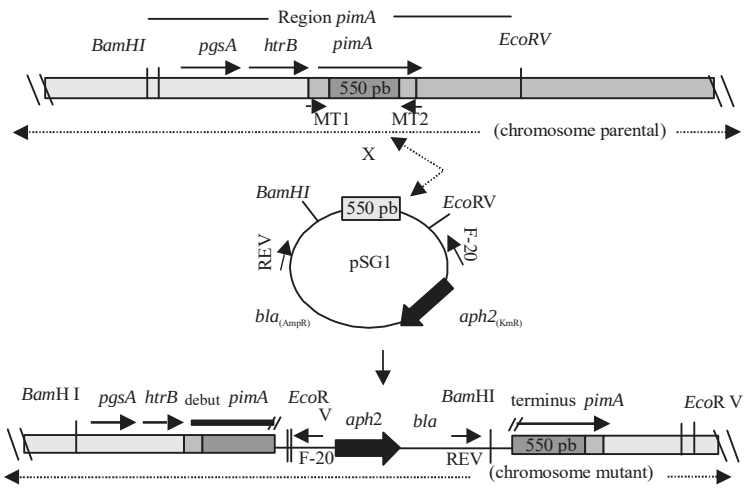

Fig. 2: Interruption of *pim*A gene of fragment *Corynebacterium glutamicum*

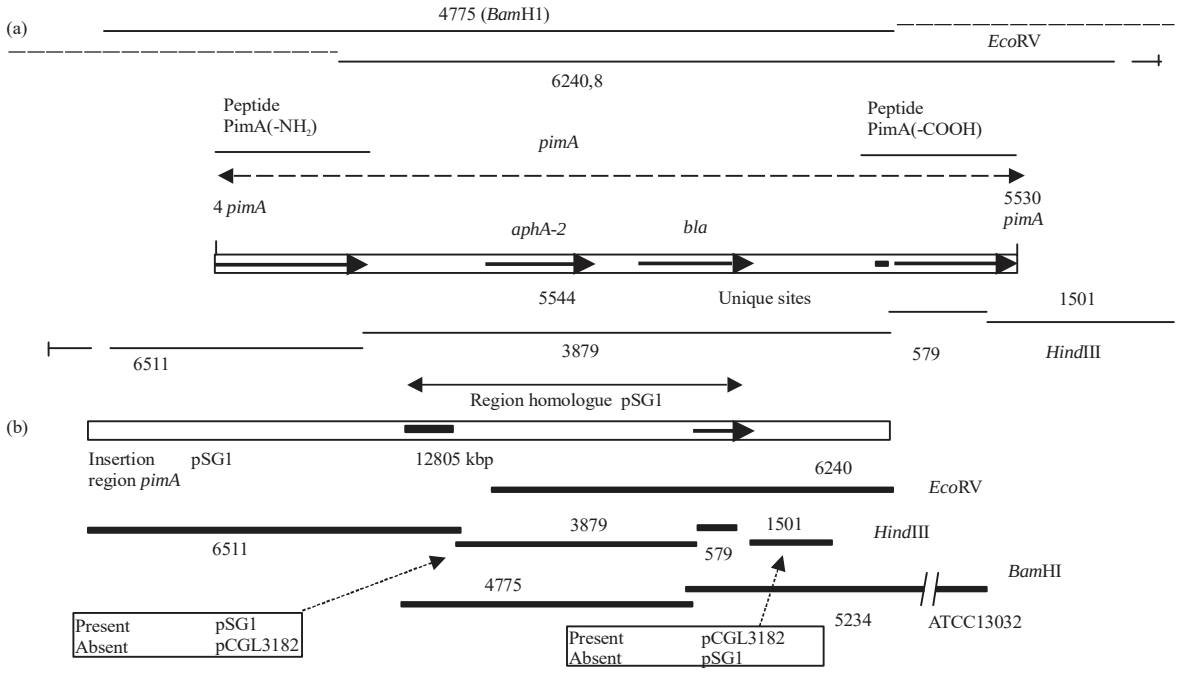

Fig. 3(a-b): (a) Integrated *pimA* allele and (b) Prediction of hybridization bands

C. glutamicum is shown in Fig. 4. A similar analysis was done by *Hind*III digestion of the DNA extracted from pSG1 transformant in the *C. glutamicum* ATCC13032 RES167 host probed by pCGL3182 revealed also the existence of an insertion in the *pim*A region (Fig. 4: channel 1). Many scientists successfully achieved mutants constructions of CMN genera. Tatituri *et al.*[32] described the disruption of PimB probable orthologue Cg-pimB and the chemical analysis of glycolipids and lipoglycans isolated from wild type *C. glutamicum* and the *C. glutamicum*::pimB mutant. Mishra *et al.*[33] also described a structural characterization and functional properties of a lipomannan variant isolated from a *C. glutamicum* pimB mutant. The construction of a *pimA* conditional mutant of *M. smegmatis* was also achieved by Korduláková *et al.*[34]. To determine whether PimA is an essential enzyme of mycobacteria, Korduláková *et al.*[34] constructed a pimA conditional mutant of *M. smegmatis* and showed that the ability of this mutant to synthesize the PimA mannosyltransferase was dependent on the presence of a functional copy of the *pimA* gene carried on a temperature-

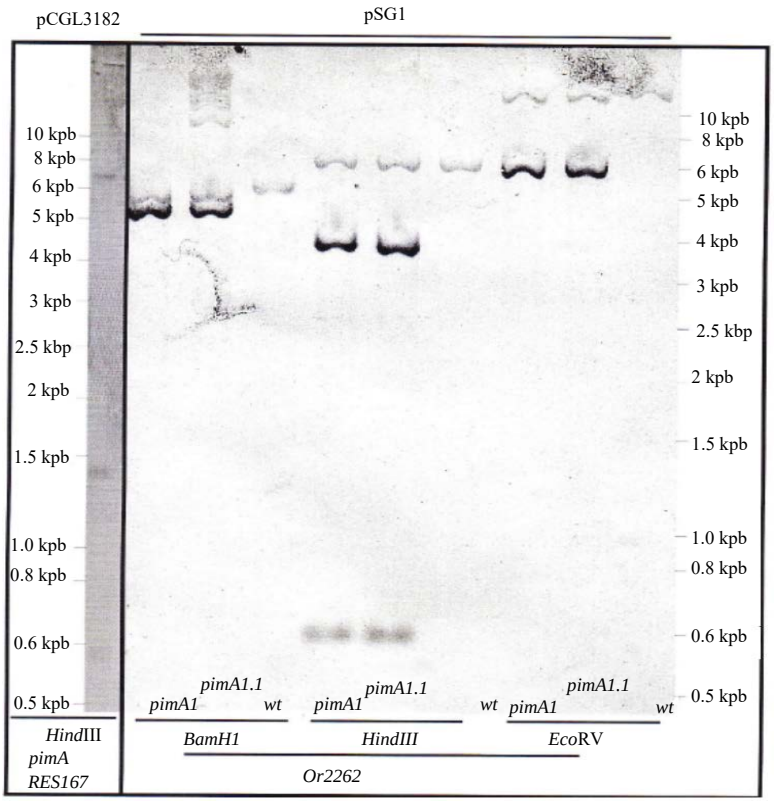

Fig. 4: Southern hybridization of *pimA* mutants DNA of *C. glutamicum*

Table 3: Characteristics of the integrative transformation in the *pimA* region

	*pim*A	
Allele		
Host	Or2262	RES167
Plasmid	pSG1	
FNTI (experience 1)	-	-
KmR-CmS		
(2 μg mL^{-1} cm)	-	-
(15 μg mL^{-1} cm)	-	-
FNTI (experience 2)	86.4[b]	570[d]
KmR-CmS		
(15 μg mL^{-1} cm)	-	-
FNTI (experience 3)	-	-
KmR-CmS		
(15 μg mL^{-1} cm)	-	-
	FTR/FTI	
	Or2262	RES167
FTR		
Strain ($\times 10^{-4}$)		
	pSG1	
Prep. a Or2262 30	-	-
Prep. b Or2262 2.13	5.3×10^{4}	-
Prep. c Or2262 46.0	-	-
Prep. d RES167 38.4	-	0.67×10^{2}

a, b, c and d refer to preparations of competent cells by electrotransformation, FTR: Frequency of replicative transformation of the batch (transformability with pCGL0243 or pCGL0609), FTI: Frequency of integrative transformation of the batch (transformability with pSG1, pCGL3181 or pCGL3182), FNTI: Normalized frequency of integrative transformation [FTI * (FTR/FMTR)]

sensitive rescue plasmid. The sequence analysis of plasmid pSG1 indicated that the insertion of the *pim*A ORF internal fragment is in line with amino-terminal extremity of the *lac*Z ORF fragment of the vector pCR2.1-TOPO (Invitrogen®, California, USA). Although, the *lac*Z gene promoter is not functional in *C. glutamicum*[35], but our results showed that there are two oriented promoters are present upstream in the same direction of *lac*Z gene. These promoters are promoters of *aph*A2 and *bla* genes of the vector which are well expressed in *C. glutamicum*.

The implications of previous results include opening the door for cloning of complete *pimA* gene of *C. glutamicum*. Cloning of complete *pimA* gene of *C. glutamicum* should make we able to analysis the whole *pgsA-htrB-pimA* region encoding for glycosyl-transferase enzyme activity of the species *C. glutamicum*. But studying the phenotypes of *pimA*, *htrB* and *pgsA* insertions is mandatory to understand the role and functionality of these genes.

CONCLUSION

PimA-like similar sequences were found to be widespread in many taxonomically related species to *Corynebacterium glutamicum*, which reflects the importance of glycosyl-

transferases enzymes activity in such species. Complete *pimA* gene of *C. glutamicum* should be cloned to be able to analysis the whole *pgsA-htrB-pimA* region. Studying the phenotypes of *pimA*, *htrB* and *pgsA* insertions is mandatory to understand the role and functionality of these genes.

SIGNIFICANCE STATEMENTS

This study discovered that cloning of an internal fragment of *pimA* gene coding glycosyl-transferase of *Corynebacterium glutamicum* is successfully achievable. The genes encoding for glycosyl-transferases enzymes activity was identified. This study will help the researchers to understand the cell wall structure of CMN genera (*Corynebacterium, Mycobacterium* and *Nocardia*) which in turn would help among others in developing more functional antibiotics against *Mycobacterium tuberculosis*.

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