CONSTRUCTION OF RECOMBINANT BCG EXPRESSING THE VP1 ANTIGEN OF ENTEROVIRUS 71 FOR THE DEVELOPMENT OF A CANDIDATE VACCINE

by

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**Thesis submitted in fulfilment of the
requirements for the degree
of Master of Science**

October 2006

ACKNOWLEDGEMENTS

Alhamdulillah, finally with Allah permission, I was successful ~~to~~ in complet~~e~~ing this ~~though~~ study. First of all, I would to express my special gratitude to my supervisor Prof. Zainul F. Zainuddin for giving me ~~a~~ the chance to be his student and also for his excellen~~ce~~t guidance in order to be an eminent scientist in the future. Special thanks for my co-supervisor, Associate Prof. Dr. Mustaffa Musa for helping me in the immunogenicity studies. All the knowledges, skills and experiences that I have ~~grabbed~~ acquired during the studies are very precious ~~and useful~~.

I would like to thank all my colleagues ~~in~~ at the School of Health Sciences, Institute for Research in Molecular Medicine and School of Medical Sciences for their support~~s~~. Without them, it ~~was~~ would have been more ~~very~~ difficult to go through this tough journey.

Last but not least, special thanks to abah, mama, abang and adik-adik fo~~u~~r your 'doa', ~~courages~~ encouragement and support~~s~~.

May Allah bless you all. Amin

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LIST OF ABBREVIATIONS

Ag85A	antigen 85A from *M. tuberculosis*
BCG	*Mycobacterium bovis* BCG (Bacille Calmete Guerin)
BCGnhC	BCG strain that carries shuttle vector backbone plasmid as a control
bp	Base pair
CD4$^+$ Th1	T helper lymphocytes
CD8$^+$ T$_c$ /CD8$^+$ CTL	Cytotoxic T lymphocytes
EV71	Enterovirus 71
HFMD	Hand, foot and mouth disease
IFN-γ	Inteferon gamma
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-13	Interleukin 13
IRES	Internal ribosome entry site

Kbp	Kilo base pair
KDa	Kilo Dalton
MHC	Major histocompatibility complex
MR	Radioactive imaging
NHpRSET	*E.coli* (BL21 DE3) strain that carries pRSET plasmid alone as a control
NHpRS-E1	*E.coli* (BL21 DE3) recombinant strain that carries pRS-E1 plasmid
rBCG	BCG recombinant strain
rBCGV1	BCG recombinant strain expresses UbGR-VP1 protein
rBCGV2	BCG recombinant strain expresses VP1 protein
Th	T-helper cell
Th1	T- helper 1
Th2	T- helper 2
TNF	Tumor necrosis factor
UbGR, UbG and UbA	Type of ubiquitin
VP1	A virus capsid protein
WIN	Pleconaril (3 - {3,5-methyl-4-[[3-methyl-(5-isoxazolyl) propyl] phenyl]-5-(trifluoromethyl)} - 1,2,4-oxadiazole)
XLA	X-linked agammaglobunemia

PEMBINAAN VAKSIN REKOMBINAN BCG YANG MENGEKSPRESKAN ANTIGEN VP1 DARI ENTEROVIRUS 71 DI DALAM PEMBANGUNAN CALON VAKSIN

ABSTRAK

Enterovirus 71 (EV71), ~~pencetus~~ penyebab wabak penyakit tangan, kuku dan mulut (HMFD) di kalangan kanak-kanak telahpun dikenalpasti sebagai wabak yang diperlu dikawal segera kerana penyakit ini telah mencatatkan bilangan kes yang tinggi sehingga sekarang. Salah satu cara yang berkesan untuk mengawal wabak ini adalah dengan kaedah pemvaksinan. Oleh itu, melalui kajian ini, calon vaksin rekombinan BCG terhadap virus EV71 telah dibangunkan. BCG rekombinan (rBCG) yang mengekspreskan gen sintetik yang mengkodkan protein VP1 daripada EV71 bercantum dengan komplek ubikuitin (UbGR) telah dihasilkan melalui teknik PCR himpunan setelah kedua-dua jujukan gen tersebut dioptimumkan mengikut kodon yang digunakan di dalam mikobakteria. Pengekspresian gen sintetik ini telah dipacu oleh promoter dan peptida berisyarat Ag85A daripada *M.tuberculosis*. Seterusnya, pengekspresian protein cantuman ~~VP1-UbGR~~UbGR-VP1 telah dikesan melalui pemblotan Western pada pellet sel rekombinan menggunakan antibodi poliklonal arnab yang spesifik terhadap VP1 protein. Imunisasi mencit BALB/c (H-2^d) dengan klon rBCGV1 menunjukkan adanya penghasilan antibodi yang signifikan di dalam serum

yang bertindakbalas dengan protein cantuman ~~VP1-UbGR~~UbGR-VP1 yang ditulenkan. Antibodi subklas IgG2a dikenalpasti mempunyai aras yang tinggi dan signifikan berbanding IgG1. Splenosit yang diperolehi daripada mencit yang diimunisasi menunjukkan peningkatan gerakbalas proliferasi limfosit apabila dirangsang oleh antigen protein cantuman ~~VP1-UbGR~~UbGR-VP1. Manakala, analis~~isa~~ sitokin intrasel yang diekspres oleh sel T CD4$^+$ dan CD8$^+$ menunjukkan protein cantuman ~~VP1-UbGR~~UbGR-VP1 merangsang sel T CD4$^+$ dan CD8$^+$ untuk mengekspreskan IL-2, IFN-γ dan IL-4~~. Antigen ini juga merangsang sel T CD8$^+$ untuk mengekpreskan IL-2 dan IFN-γ~~. Analis~~isa~~ sitokin ekstrasel menunjukkan aras IFN-γ yang tinggi dan signifikan apabila splenosit bertindakbalas dengan antigen ~~VP1-UbGR~~UbGR-VP1. Secara keseluruhan, keputusan analis~~isa~~ ujian imuniti ke atas mencit mencadangkan bahawa calon vaksin rBCGV1 dapat merangsang tindakbalas imuniti Th1 (T-helper 1) terhadap antigen yang dikaji. Keputusan-keputusan kajian ini menunjukkan rBCGV1 berpotensi dibangunkan sebagai vaksin dan kajian yang berterusan diperlukan bagi memastikan keberkesanannya.

CONSTRUCTION OF RECOMBINANT BCG EXPRESSING THE VP1 ANTIGEN OF ENTEROVIRUS 71 FOR THE DEVELOPMENT OF A CANDIDATE VACCINE

ABSTRACT

Enterovirus 71 (EV71), the causative~~ing~~ agent of outbreaks of hand, foot and mouth disease (HFMD) in children ~~is identified as a current outbreak that needs~~require urgent control due to the ~~higher~~ high number of cases. Vaccination is one of the most effective methods to control ~~the~~ disease outbreaks~~s~~. In this study, a recombinant BCG vaccine candidate was constructed against EV71. The recombinant BCG (rBCGV1) ~~which~~ expresses~~ed~~ a synthetic gene encoding the~~ed~~ VP1 protein of EV71 fused to ubiquitin complex (UbGR) which was constructed~~amplified~~ using the technique of assembly PCR. The synthetic gene was codon ~~technique after~~ optimized for~~ing the genes into~~ expression mycobacterium~~codon bias~~. The Ag85A promoter and signal ~~peptide~~ peptide sequence from *M.tuberculosis* ~~drove~~ was used to drive the expression and secretion of the synthetic gene~~expression was from *M.tuberculosis*~~. ~~Hence, T~~the expression of the ~~VP1-UbGR~~UbGR-VP1 fusion protein was ~~revealed~~ confirmed by Western blotting using rabbit polyclonal antibody specific to the VP1 protein and was found in the cell ~~recombinant~~ pellet ~~cell by the used of~~of the

recombinant BCG showed the ability to induce moderate antibody production in BALB/c (H-2d) mice when sera from immunized mice were tested against purified UbGR-VP1 fusion protein. IgG2a subclass antibody was shown to be induced at a significantly higher level than IgG1. Splenocytes obtained from rBCGV1 immunized mice showed significant higher level of lymphocyte proliferation when stimulated with UbGR-VP1 compared to control. Analyses of intracellular cytokines show that CD4$^+$ T cells and CD8$^+$ T cells from rBCGV1 immunized mice were stimulated by UbGR-VP1 protein to express significant leves of IL-2, IFN-γ and IL-4 when compared to the control. The extracellular cytokine analysis also showed significantly higher levels of IFN-γ compared to control. Overall, the immunogenicity studies results suggested that the rBCGV1 enhanced the stimulation of immune system towards the Th1 (T helper 1) pathway. Data from this study also suggested the potential of rBCGV1 to be developed as a vaccine and further studies must be carried out to evaluate the efficacy of this candidate vaccine.

CHAPTER 1

INTRODUCTION

1.0 Background

Enterovirus 71 (EV71) is a human Enterovirus A belonging to the Enterovirus genus classified in the Picornaviridae family. Since the first outbreak of EV71 in 1969, epidemics have occurred periodically throughout the world (Takomoto *et al.,* 1998 and Hagiwara *et al.,* 1978). The most common manifestation observed in EV71 infected patients is childhood exanthema or also known as hand-foot-and-mouth-disease (HFMD) (McMinn, 2002). EV71 can also cause neurological disease during the acute infection case. The most severe clinical symptoms of EV71 infection associated with neurological disease are aseptic meningitis, brainstem and/or cerebellar encephalitis, and acute flaccid paralysis (AFP). Children less than 5 years of age are vulnerable to EV71 infection. Neurological complications of the infection occasionally cause permanent paralysis or death. In the Asia Pacific region, several large outbreaks of EV71 epidemics have been reported in young children with fatal brainstem encephalitis leading to high number of fatalities (Cardosa *et al.*, 1999, Ho *et al.*, 1999, Komatsu *et al.*, 1999 and Lum *et al.*, 1998). In Malaysia, 34 children were reported dead during the EV71 outbreak in Sarawak (Cardosa *et al.,* 1999). To control these epidemics, the development of effective candidate vaccines against EV71 is important (McMinn, 2002).

1.1 History of EV71

In 1969, the first EV71 outbreak was reported in California where the virus from the stool of the infants with encephalitis (Schmidt *et al.,* 1974). EV71 associated with severe neurological disease cases were later isolated in New York between 1972 and 1977 (Melnick, 1984). The first EV71 infection outside of the USA was reported in

Melbourne, Australia during on epidemic of aseptic meningitis between 1972 and 1973 (Kennett *et al.*, 1974). In 1973, the first outbreak of HFMD was observed in Sweden (Blomberg *et al.*, 1974) and Japan (Hagiwara *et al.*, 1978).

In Europe, the first reported case of EV71 occurred in Bulgaria (1975) with the large and severe epidemics of encephalitis and AFP (Nagy *et al.* 1982 and Chumakov *et al.*, 1979). 705 cases of EV71 were documented in the Bulgarian epidemic. Of that, 83.8% of the total cases occurred in children under 5 of years age and 93% were paralytic cases. Children with bulbar disease (AFP) were reported dead between 10 and 30 hours after onset of illness (Chumakov *et al.,* 1979).

In the Asia Pacific region, EV71 cases were identified in Japan between 1973 and 1978 with low prevalence of acute neurological disease (Hagiwara *et al.*, 1978). In 1985, a small outbreak of EV71 occurred in Hong Kong with several cases of AFP (Samuda *et al.*, 1987). An outbreak was reported in Hubei Province, China during the winter of 1987 (Zheng *et al.*, 1995). Several large epidemics and high-level endemic circulation have been identified since 1997 such as outbreaks in Japan (1999) (Komatsu, *et al.,* 1999) and Singapore (1998) (Singh *et al.,* 2000). In 1997, the first epidemic of EV71 in Malaysia occurred in Sarawak (Cardosa *et al.*, 1999 and WHO, 1997) and later in Peninsular Malaysia in 2001 (Lum *et al.,* 2002). In Malaysia, numerous cases of HFMD, herpangina and neurological complications including aseptic meningitis, AFP and cerebellar ataxia were reported in young children. In addition, the signal feature of the epidemic in Sarawak was the syndrome of rapidly fatal neurogenic pulmonary oedema associated with severe brainstem encephalitis, that caused 34 fatalities (Cardosa *et al.*, 1999). In Peninsular Malaysia, 4 fatal cases were reported associated with this syndrome (Lum *et al.*, 2002).

The largest EV71 outbreak was reported in Taiwan in 1998 (Liu *et al.*, 2000, Hsiung and Wang, 2000 and Ho *et al.*, 1999) which occurred in two waves. The first occurred between March and July involving the whole island and the second outbreak occurred from September to November which was confined to the southern region. Approximately 3.6 million cases were reported. 1.5 million HFMD cases were estimated to occur during this epidemic by the National Sentinel Surveillance Network and 2.1 million cases were asymptomatic (Ho, 2000a). Four hundred and five cases of severe neurological disease due to EV71 infection were documented and 78 cases were fatal due to neurogenic pulmonary oedema (Lum *et al.*, 2002, McMinn *et al.*, 2001a and Hsiung and Wang, 2000).

In 1999, a total of 6000 cases of HFMD occurred over a six months period from March until August and 29 cases progressed to severe neurological disease during the large outbreak in Perth, Australia (McMinn *et al.*, 2001b). The EV71 epidemic continued to circulate in the Asia Pacific region during the year 2000 to 2001. This endemic occurred again with sporadic cases of HFMD and encephalomyelitis (Cardosa, 2006). Several fatal cases of encephalomyelitis were documented in Singapore and Peninsular Malaysia respectively in the year 2000 (Lum *et al.*, 2002). In 2001, numerous cases of HFMD and severe encephalomyelitis were reported in Southeastern Australia (McMinn, 2002).

1.2   EV71 epidemiology and evolution

A molecular epidemiological study of EV71 was done by Hagiwara *et al.,* (1984) to determine the variations between EV71 strains by analyzing the capsid's polypeptide and RNase T1 sequences of the virus isolated from different regions such as Japan, Taiwan, Bulgaria, Hungary and USA. However, these methods were insufficient to identify specific EV71 genetic lineages or molecular markers of virulence.

The 5' untranslated region (5' UTR) of EV71 has been studied in China by comparing nucleotide sequence (Zheng *et al.*, 1995). Significant genetic variations between EV71 strains from different locations or time of isolation were found using this technique.

Brown *et al.,*(1995) contributed important information on the molecular epidemiology and evolution of EV71. The complete VP1 sequences of 113 EV71 isolates from all over the world were compared in their study. Three independent genetic lineages (genogroups) of EV71 (A, B, C) were identified and were shown to evolved over 30 years. More than 92% of nucleotides sequences were identical within same genogroup whereas 78 to 83% identities were found between genogroups. The EV71 BrCr strain was the only strain classified into genogroup A, whereares sub-lineages such as B1/B2 and C1/C2 were classified in genogroups B and C respectively.

Virus capsid protein (VP1) gene sequence of 18 strains from the 1998 Taiwanese epidemic including all strains isolated from fatal cases showed that the majority of these isolates belong to a new lineage within genogroup C2 (Shih *et al.*, 2000). This finding was correlated with studies done by McMinn *et al.,* 2001a.

In McMinn *et al.,* 2001a studies, the complete VP1 gene sequence of 66 EV71 strains isolated in Malaysia, Singapore, Taiwan and Western Australia were compared. The study showed that two previously unidentified lineages within genogroup B (B3 and B4) found circulating in Southeast Asia between 1997 and 2001 were also later confirmed as similar to the new strain found in theTaiwanese epidemic (McMinn *et al.*, 2001a).

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Genogroup B3 was shown to be predominantly in South East Asia and was reported as the major cause of epidemics in Sarawak (1997), Singapore (1998) and Western Australia (1999) (McMinn *et al.*, 2001a). Meanwhile, genogroup B4 was found in a small number of cases in Singapore (1997), Peninsular Malaysia (1997 to 1998) and Taiwan (1998), indicating that genogroup B4 was widespread in Asia Pacific region but was not predominant. In 2000, genogroup B4 became the cause of a large epidemic in Malaysia (Peninsular and Sarawak) and Singapore, apparently replacing genogroup B3 viruses (McMinn *et al.*, 2001a).

Virus strains belonging to single lineages within genogroups B3 and C2 were identified during the Western Australia epidemics in 1999 (McMinn *et al.*, 2001a). Nucleotide analysis, show that those of the genogroups B3 and C2 were highly similar with the genogroup C3 found in Sarawak (99%) and genogroup C2 found in Taiwan (98%) respectively.

A phylogenetic study of EV71 strains based on the VP4 gene sequence has been done in various countries (Shimizu *et al.*, 1999). The results, suggest that EV71 strains from Bulgaria and Hungary belong to genotype A-1 whilst those from Malaysia were identified as a genotype A -2. Strains in genotype B were more prevalent in Japan and Taiwan. The study also showed that the European strains were distantly related to recent 'VP1-based' genogroup B3 and B4 isolates from the Asia Pacific region (Shimizu *et al.*, 1999).

1.3   The EV71 virus

EV71 belongs to the human Enterovirus A species and is classified under the enterovirus genus within the Picornaviridae family. This virion consists of a non-enveloped capsid surrounding the core of 7.5kb single stranded RNA which has

positive polarity. The viral capsid is icosahedral in symmetry and is composed of 60 identical units (protomers), each consisting of 4 structural proteins VP1, VP2, VP3 and VP4, respectively.

The complete nucleotide sequence of the EV71 prototype strain BrCr has been determined. The single open reading frame (ORF) encodes a polyprotein of 2194 amino acids and is flanked by untranslated regions (UTRs) at the 5' and 3' ends. A variable length poly-A tract is located at the terminus of the 3' UTR. The polyprotein is subdivided into three regions, P1, P2 and P3, respectively. The P1 region encodes 4 viral structural proteins named as 1A-1D (VP1-VP4). Meanwhile the P2 and P3 regions encode non-structural proteins known as 2A-C and 3A-D, respectively. The functions of the 11 individual EV71 proteins are thought to be similar to that found in poliovirus and other non-polio enterovirus (Brown and Pallansch, 1995).

1.4 Molecular genetics of virulence in EV71

Neurological manifestations are important aspects in EV71 infections to determine the neurovirulence determinants in EV71. Many studies have been done and were mainly focused on the VP1 gene and its product as well as the 5'UTR region.

1.4.1 The VP1 gene and protein

The VP1 gene in the EV71 genome has been identified as the most informative region to study the evolutionary relationships of EV71 strains. The VP1 protein has a highly conserved tertiary structure and has been identified as the source of virulence determinants for several enteroviruses (Caggana et al., 1993). The VP1 protein is also thought to be the major viral neutralization determinant and has a high degree of antigenic and genetic diversity. A deep cleft on the virion surface has been identified at the junction of VP1 and VP2 and is thought to function as the site of virion attachment to

the cellular receptor (Blomberg *et al.*, 1974). No homologous recombinations have been shown to occur in the VP1 gene. Thus, the VP1 gene has been found to be the most useful in distinguishing within and between enteroviruses serotypes (Oberste *et al.*, 1999 and Oberste *et al.*, 1995).

McMinn *et al.,* (2001a) discovered that an EV71 strain in genogroup C2 of the Perth epidemic has a mutation at codon 170 causing a change from alanine to valine. The mutation was discovered through comparative analysis of the Perth VP1 deduced amino acid sequence with the VP1 consensus amino acid sequence of other EV71 strains (genogroups A, B, C and CA16). The genogroup C2 viruses were isolated from children with severe neurological disease. By contrast, this substitution mutation (alanine-valine) in position 170 was not found in viruses obtained from the numerous cases of uncomplicated HFMD (McMinn *et al.,* 2002 and McMinn, 2001a). The alanine residue at position 170 is a highly conserved region and exists in all EV71 and CA16 strains. The substitution of alanine to valine increases hydrophobicity at this site and may change the protein conformation (Garnier *et al.,* 1978). These data suggest that the mutation may be associated with the increased neurovirulence in the Perth epidemic (McMinn *et al.,* 2001b).

The amino acid (mutation alanine to valine) at position 170 is located in an α-helix structure within the E-F loop in VP1, at the interface between the protomer subunit and on the rim of the canyon. This region that may play a role in uncoating following receptor binding (Garnier *et al,* 1978). In VP1 poliovirus protein, mutations at the E-F loop have been shown to alter receptor specificity by a mechanism thought to involve conformational change within the cleft (Wien *et al.*, 1997 and Filman *et al.*, 1989). The Garnier-Osguthorpe-Robson equation for secondary structure analysis was used by Garnier *et al.,* (1978) to study the alanine to valine substitution and the results

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suggest that the change was likely to alter the protein conformation in the E-F loop from an α-helix to a β-sheet.
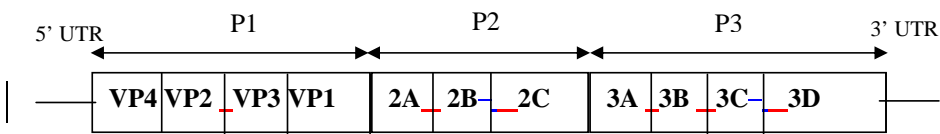
Fig 1.1: EV71 genome structure.The ORF is divided into three regions: the P1 region encodes four structural proteins VP1-VP4; the P2 and P3 regions encode seven non-structural proteins 2A-2C and 3A-3D, respectively. Adapted from Brown and Pallansch (1995).
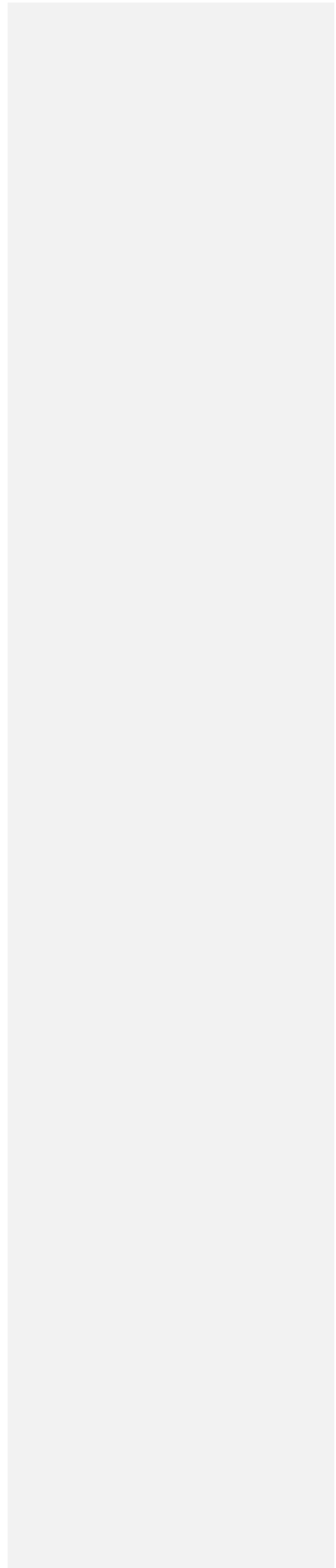
1.4.2 The 5' UTR region

The 5' UTR of EV71 contain a group of conserved secondary structure element which is called the internal ribosome entry site (IRES). The IRES regulates enterovirus replication through the control of cap-independent translation of the polyprotein. Within the 5' UTR of EV71, IRES-like stem loop structure has been identified (Abubakar *et al.*, 1999). A single nucleotide change within the poliovirus IRES resulted in a large alteration of the neurovirulence of the virus (Evans *et al.*, 1985). Although, the EV71 IRES may play an important role in neurovirulence its actual role remains unclear because nucleotide variation linked to neurovirulence in the EV71 IRES has not been identified to date.

```
                    150         160         170         180         190         200
                     |           |           |           |           |           |

CA16                lqymyvppgapkptsrdsfawqtatnpsifvkltdppaqvsvpfmspasa

EV71 consensus      lqymyvppgapkpesresfawqtatnpsivfvkltdppaqvsvpfmspasa

A (BrCr)            --------------d----p-----------ss-----------------

B consensus         --------------------------------------------------

C consensus         -------------d------------------s-----------------

27M/AUS/99          -------------d------------------s-----------------  ⎫

2M/AUS/99           -------------d---------v--------s-----------------  ⎬

5M/AUS/99           -------------d---------v--------s-----------------  ⎪ Perth
                                                                          (1999)
6F/AUS/99           -------------d---------v--------s-----------------  ⎪ lineage 1

8M/AUS/99           -------------d---------v--------s-----------------  ⎭

9F/AUS/99           -------------d---------v--------s-----------------

7F/AUS/99           -------------d------------------s-----------------  ⎫ Perth
                                                                          (1999)
14F/AUS/99          -------------d------------------s-----------------  ⎭ lineage 2

NCKU9822            -------------d------------------s-----------------
```
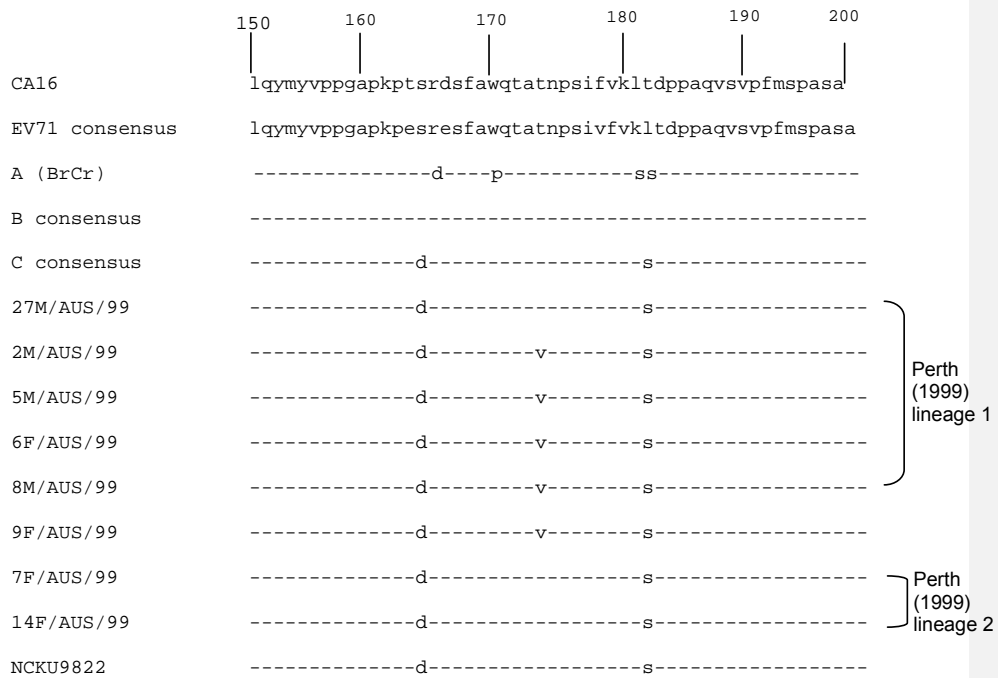
Fig. 1.2: Partial alignment of VP1 deduced amino acid sequence (residue 150-200) of the EV71 isolates from Western Australia belonging to genogroup C2. The mutation alanine to valine appeared in lineage I (residue 169). Adapted from McMinn, 2002.

1.5   Clinical symptoms of EV71 infection

1.5.1 Hand foot and mouth disease (HFMD) and Herpangina

HFMD have been associated most frequently with Coxsackievirus 16 (CA16), but also has been recognized in other enterovirus serotypes including EV71, CA5, CA9 and CA10 (Melnick, 1984). This illness is characterized by 3 to 4 days of fever and the development of vesicular exanthem on the buccal mucosa, tongue, gums and palate and a papulove-sicular exanthema on the hand, feet and buttock (McMinn *et al.*, 2001b and Komatsu *et al.*, 1999). The rashes of HFMD due to CA16 and EV71 may differ (McMinn *et al.*, 2001b and Komatsu *et al.*, 1999). The size of the CA16 infection vesicles is larger than those in EV71 infections. In EV71 infection, the rash is more frequently papular and/or petechial, often with areas of diffuse erythema on the trunks and limbs.

Herpangina is an illness characterized by an abrupt onset of fever and sore throat, associated with the development of raised papular lesions on the mucosa of the anterior pillars of fauces, soft palate and uvula (Hayward *et al.*, 1989).

1.5.2 Neurological disease

EV71 has been shown to be highly neurotropic and associated with a diverse range of neurological diseases such as aseptic meningitis, brainstem and/or cerebellar encephalitis, AFP and several post-infectious neurological syndromes (Schmidt *et al.*, 1974).

AFP associated with EV71 infection appears to be milder with higher rates of complete recovery compared to AFP due to poliovirus infection (McMinn *et al.*, 2001b, Hayward *et al.*, 1989 and Samuda *et al.*, 1987). EV71 may induce AFP by several mechanisms in addition to virus-mediated destruction of the anterior horn motor neurons. This is reflected in the more varied clinical presentation of EV71-associated AFP than that seen during poliovirus infection (Alexander *et al.,* 1994).

Brainstem encephalitis is the most severe neurological manifestation of EV71 infection. Radioactive MR imaging and post-morterm studies indicate that the medulla oblongata, reticular formation, pons and midbrain structures are most frequently involved (Lum *et al.*, 2002, McMinn *et al.*, 2001b and Huang *et al.*, 1999). Myoclonus, tremor, ataxia, nystagmus and cranial nerve palsies have been presented in infected children (Huang *et al.*, 1999 and Wang *et al.*, 1999).

Acute cerebellar ataxia has been associated to EV71 in many previous epidemics (McMinn *et al.*, 2001b, Komatsu *et al.*, 1999, Nagy *et al.*, 1982 and Ishimaru *et al.*, 1980). A study of MR imaging of two cases of EV71 associated cerebellar ataxia indicated that the acute disease was linked to inflammation of the grey matter in both cerebellar hemispheres and variable atrophic changes of the cerebellar cortical atrophy may develop upon recovery, despite complete recovery of function (McMinn *et al.*, 2001b).

1.5.3 Neurogenic pulmonary oedema

Neurogenic pulmonary oedema, also known as the grade III brainstem encephalitis has been associated with a rapid onset of cardiopulmonary failure (Huang *et al.*, 1999). The fatality rate is 80% and all surviving children developed significant neurological sequeale during an epidemic in Taiwan(1999). Typically, children

develops tachycardia, tachypnoea and cyanosis between two and five days after the onset of fever, HFMD or herpangina (Lum *et al.*, 2002 and Ho, 2000b).

1.6   Transmission of EV71

The mode of transmission of most enteroviruses including EV71 is by the faecal-oral-route via fecally contaminated materials. EV71 survive well in the external environment and are resistant to heat, desiccation and low temperature. The incubation period is characteristically 3 to 5 days between infections (Ho, 2000a). The initial sites of the EV71 replication are at the pharynx and terminal ilium (Fig.Figure 1.3). Virus is shed from the upper respitory tract for 1 to 3 weeks and from the feces for up to 38 weeks. In an epidemic in Taiwan, the risk factors of transmission of EV71 infection were identified as positive serology for older sibling, children between 6 months and 3 years of age, the number of children in the family and history of HFMD or herpangina (Ho, 2000a).

DAY:

0 — Small Intestine: Primary infection, replication

1 — Mesenteric lymph nodes: Replication

2 — Bloodstream: Primary viraemia

3 — Central focus of replication

4

5 — Initial appearance of antibodies

6 — CNS: Infection, replication, intraneural spread

7

8

9

10 — High level of antibody in serum

11 — Paralysis

12 — Excretion of virus in faeces
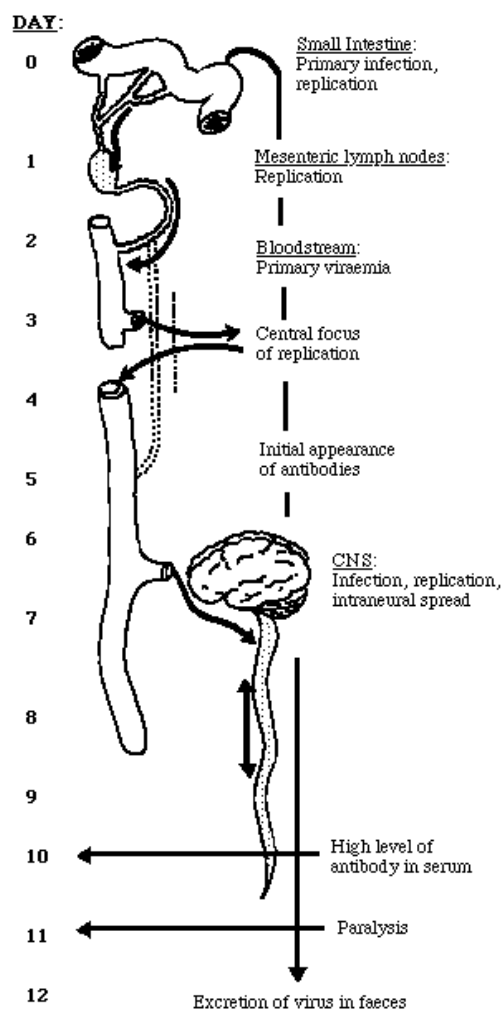
Fig.Fig. **1.3:** Replication and dissemination route of EV71 infection in human body.

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(Adapted
from www.vadscorner.com/outbreak.html)

1.7   Immunity of EV71

1.7.1 Cell-mediated immunity

Cell-mediated immunity is the main key component involved in host defense against most viruses including the enteroviruses. The main task of cell-mediated immunity is to kill virus-infected cells. Cytotoxic T lymphocytes CD8$^+$ Tc (CTL) cell and T helper lymphocytes CD4$^+$ Th1 cells are the main components of cell mediated antiviral defense (Germain, 1999). Activated Th1 cells produce a number of cytokines, including Interleukin 2 (IL-2), Interferon gamma (IFN-γ) and tumor necrosis factor (TNF) that defend against viruses either directly or indirectly. IFN-γ enhances the antiviral activity of macrophages. Meanwhile, IL-2 acts indirectly by assisting the recruitment of CTL precursors into effector population. Both IL-2 and IFN-γ activate NK (Natural Killer) cells, play an important role in host defense during the first days of many viral infections until a specific CTL response develops. Specific CTL activity arises within 3 to 4 days after infection, peak by 7 to 10 days, and then gradually decline. Within 7 to 10 days of primary infection, most viruses have been eliminated, parallelling the development of CTLs.

In general, CTLs recognize antigens as short fragments of proteins (8 to 10 amino acid length) bound to the major histocompatibility complex (MHC) class I molecules on the cell surface. Most of these peptide epitopes are derived from the cytosolic degradation of proteins that are synthesized within the host cell and are

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translocated by specific transporters from the cytosol into the endoplasmic reticulum where they bind to MHC class I molecules. The peptide-MHC class I complex then is transported, through the Golgi apparatus, to the cell surface where it is presented to CD8$^+$ T lymphocytes.

1.7.2 Humoral immunity

Humoral immunity plays a key role in neutralizing extracellular pathogens and therefore is crucial for the clearance of the cytopathic viruses to prevent the reinfection. In general, antibodies produce in viral infection including Immunoglobulin M (IgM), IgA and IgG. Initially, IgM will be produced in enterovirus infections including EV71 (Sheng *et al.,* 2004). Patients with X-linked agammaglobunemia (XLA) who have been infected by enteroviruses, have the normal profile of cell mediated immunity but are defective in humoral immune response (Rotbart *et al.*, 1998). This feature leads to chronic enterovirus meningoencephalitis with fatal outcome. This suggests that humoral immunity plays an important role in the body defense against enterovirus infection.

1.8 Diagnosis of EV71 infection

The standard diagnostic approach that has been used in detecting EV71 is by viral isolation from patient specimens including feces, throat swabs, rectal swabs, cerebrospinal fluid or vesicular fluid. The specimens are inoculated into Vero, RD or HeLa cell cultures and the cells are incubated for a least a week. Cultures exhibiting a cythopathic effect are identified by microneutralization assay with antiserum against EV71, or by an indirect immuno-fluorescent assay with monoclonal antibody against BrCr strain (Shimizu *et al.*, 1999). However, this method is time consuming and relies on the availability of the antisera or commercial monoclonal antibodies.

To avoid these time-consuming methods, the IgM-capture enzyme linked-immunosorbant assay (ELISA) was developed for rapid detection of EV71 infection (Sheng *et al.,* 2004 and Kuo *et al.,* 2002). The sensitivity and specificity of the assay were 97.7% and 93.3% (Sheng *et al.*, 2004). Viral isolation and neutralization tests were used as standards. With this assay, the EV71 infection can be detected within 4 hours.

Shin *et al.,* (2000) established the Western blot method as a diagnosis tool by using the VP1 capsid protein of EV71 as an antigen. Using the assay, anti-VP1 IgM appeared in sera of patients with a symptomatic acute infection, while anti-VP1 IgG was observed in sera of patients with past infection. These findings suggest that detecting IgG and IgM immune responses against VP1 antigens are an effective means of determining the different phases of EV71 infection.

Various molecular assays were also developed to detect EV71 infection, these included reverse transcriptase PCR (RT-PCR) and DNA microchip arrays (Shin *et al.,* 2003, Kuo *et al.,* 2002 and Singh *et al.,* 2000). RT- PCR was established by Kuo *et al.,* (2002) to detect coxsackie A16 and EV71 which induces HFMD. Meanwhile, a DNA microchip assay for EV71 detection was developed by Shin *et al.,* (2003) as an alternative diagnostic method and takes 6 hours. One hundred specimens were identified as positive for enteroviruses by viral culture including 67 EV71 specimens and these were tested using this microchip. The sensitivity and specificity of this novel method was 89.6% and 90.9% respectively.

1.9 Control of EV71

1.9.1 Antiviral agents

Much work have been done to develop the promising anti-enterovirus agents but few are, close to commercial release and their effects on EV71 are still being

investigated. The most promising agent and which are still under clinical trials are the WIN group of compound. The WIN compound, pleconaril (Rotbart *et al.,* 1998) has been found to provide significant therapeutic benefit in aseptic meningitis, AFP and encephalitis due to many enteroviruses serotypes (Rotbart and A.D., 2001 and Starlin *et al.,* 2001). Plenocaril (3 - {3,5-methyl-4-[[3-methyl-(5-isoxazolyl) propyl] phenyl]-5 (trifluoromethyl)} - 1,2,4-oxadiazole) works by inhibiting the uncoating and the release of infectious viral RNA and this action inhibits the production of virions progeny.

The IVIG (intravenous immunoglobulin) or anti-inflammatory agent has been given to patients with X-linked agammaglobulinemia (XLA) and enterovirus meningoencephalitis. These patients fail to produce neutralizing antibody against enteroviruses infection. In the case of patients with chronic enteroviral meningoencephalitis, the strategy was successful. Enteroviral replication was suppressed and the cerebrospinal fluid (CSF) cultures were shown to be negative following treatment (Mease *et al.*, 1981).

Even though these antiviral agents have high-level activity against EV71, but they still need to be further clinically evaluated. Thus, research efforts must be focused on the prevention of EV71 infection through the development of effective vaccines.

1.9.2 Vaccines

To prevent epidemics of EV71, the development of effective vaccine candidates are required for protection. Previously, a formalin-inactivated EV71 vaccine had been developed to control the Bulgarian epidemic in 1975, but has not been used since. Furthermore, no available data on the efficiency of this vaccine (Nagy *et al.*, 1982 and Chumakov *et al.*, 1979).

Cheng *et al.,* (2001), constructed three candidate EV71 vaccines; a formalin-inactivated vaccine, a VP1 DNA subunit vaccine and a recombinant VP1 subunit

protein vaccine. In challenge studies of these vaccine candidates, mice groups vaccinated with the inactivated virus vaccine showed 80% survival with a challenge dosage of 2300 LD_{50} virus/mice. The subunit vaccines provided protection only at a lower challenge dosage of 230 LD_{50} virus/mouse, with 40% survival for DNA vaccine and 80% survival for the protein recombinant vaccine. The cytokines profiles produced by splenocytes showed high levels of interleukin 4 (IL-4) in the inactivated virus vaccine group, high levels of IFN-γ and interleukin 12 (IL-12) in the DNA vaccine group and high levels of interleukin 10 (IL-10) and IFN-γ in the protein recombinant vaccine group. Overall, the inactivated virus vaccine elicited a much greater magnitude of immune response as compared to the subunit vaccines, including total IgG subtypes and T helper cell responses. These data indicated that the inactivated EV71 virus vaccine is the best vaccine preparation capable of fulfilling the demand of effective control, and that VP1 subunit vaccines remain promising strategies for the development of EV71 vaccine. Suh *et al.*, (2004) have optimised the micro carrier cell culture process to produce the inactivated EV71 virus vaccine.

The antigenic epitopes of EV71 involved in protection remain unknown. Thus, the identification of such epitopes is a major priority in vaccine research, as it will allow the development of transgenic mouse model for studies of EV71 pathogenesis and for the development of more effective vaccines (McMinn, 2002).

1.10   Potential of recombinant BCG as a candidate vaccine against EV71 infection

Bacille Calmette Guerin (BCG) has the potential to be developed as a vehicle for delivering foreign antigens by using recombinant DNA technology (Hetzel *et al.,* 1998 and Stover *et al.,* 1991). This technology is based on the transfer of a selected gene encoding an antigen capable of inducing productive immune response, to a non-pathogenic host, thereby making the production of an antigen safer and more efficient.

This approach has been used for the development of a few vaccines candidates such as against HIV (Human immunodeficiency virus type 1) (Aldovini and Young, 1991) and, malaria (Norazmi and Dale, 1997). and tuberculosis (REF?).

BCG has been selected to deliver several viral antigens in a number of studies because of it's unique advantages and suitability as a multivaccine vehicle. The advantages include the following:

1) BCG is the most widely used vaccine in the world, having been given to over 2500 million people since 1948, with low incidence of serious complications.

2) It can be administered at or any time after birth, and is unaffected by maternal antibodies.

3) BCG is given as a single dose

4) It is a potent adjuvant in experimental animals and human

5) BCG can be administered as an oral vaccine

6) It is the most heat stable of live vaccines

7) It is inexpensive to produce

A number of researchers have expressed viral epitopes in BCG and were able to induce immune response against these epitopes. Aldovini and Young (1991), found that rBCG which expressed the viral epitopes of HIV Gag, Pol and Env polyprotein under the control of heat shock protein promoter Hsp 70 can induce antibody production and T-cell responses against these epitopes in mice. The same approach also been used by Yasutomi *et al.,* (1993) to express the antigen *gig* of simian immunodeficiency virus of rhesus monkey (SIV mac 251). Immunization with this rBCG elicited MHC class I-restricted, CD8+ SIV-mac251 gag-specific CTL in rhesus monkey.

1.11 Ubiquitin

Ubiquitin is a 76 amino acid peptide involved in controlling normal protein intracellular turnover in the cytoplasm of eukaryotic cells. Proteins that will be degraded are tagged by ubiquitin molecules through covalent attachment and presented to the proteosome chamber. In the proteosome, the target proteins will be degraded into small fragments of peptides by proteolytic enzymes. The conjugation of ubiquitin has been shown to enhance the cell mediated immune response in vivo against the antigen (Rodriguez *et al.,* 1997 and Tobery and Siliciano, 1997).

In general, when the cells are infected by virus, the viral proteins will be conjugated to ubiquitin and transferred into proteosomes. After the degradation process, the small peptide fragments will be released and transported to MHC class I receptors on the surface of the cells. CD8$^+$ CTLs will recognize the MHC class I molecule associated with the viral fragment complex and the activated CTLs will lyse the infected cells before the new virions are produced.

Rodriguez *et al.,* (1997) constructed a DNA vaccine that encodes an antigen of the lymphocytic chorionmeningitis virus (LCMV) nucleoprotein (NP) fused to mouse ubiquitin. Vaccination of this DNA vaccine in mice, resulted in the enhancement of cytotoxic T-lymphocyte (CTL) induction and antiviral protection. However, this vaccine does not appear to induce antibodies. Similar findings were reported by Tobery and Siliciano (1997) who constructed and evaluated recombinant vaccinia vectors encoding an HIV-1 antigen that has been tagged to ubiquitin.

Delogu *et al.,* (2002) successfully developed three versions of DNA vaccines encoding the MPT64 tuberculosis antigen fused to different variants of ubiquitin (UbG, UbA and UbGR). These ubiquitin proteins are known to differentially affect the intracellular processing of the co-expressed protein. Vaccination of the 3 DNA candidate vaccines (designated as UbG64, UbA64 and UbGR64) in mice gave different

results of the immune response. The UbA64 DNA vaccine induced a weak humoral response as compared to UbG64, and a mixed population of IL-4 and IFN-γ secreting cells. Vaccination with the UbGR64 plasmid generated a strong Th1 cell response (high IFN-γ and low IL-4) in the absence of any detectable humoral response. Thus, the expression of the antigen (MPT64) fused to UbA or UbGR molecules, shifted the host towards a stronger Th1 immunity response which was characterised by low specific antibody level and high number of IFN-γ secreting cells. Mice immunized with the UbGR64 DNA vaccine showed resistance to subsequent challenge with *M. tuberculosis*. Therefore, ubiquitins appear to be useful in directing towards a particular type of immune response and has potential for use in vaccine development.

1.12   The aims of study

The main goal of this study is to explore the use of live recombinant *Mycobacterium bovis* BCG as candidate vaccines against EV71 infection. In addition, the use of UbGR (Delogu *et al.,* 2002) as a tool to enhance immune response will also be explored.

The main aims of the study are:

1) To generate a synthetic Mycobacterium codon optimized VP1 gene of EV71 fused to ubiquitin (UbGR) by assembly PCR.

2) To clone the synthetic gene into a shuttle vector containing the Ag85A *M.tuberculosis* promoter for protein expression in BCG.

3) To test and evaluate the immunogenicity and safety of the rBCG candidate vaccines in a mouse model.

(See flow chart at Fig.Figure 1.45)

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┌─────────────────────────────┐
│ Production of the synthetic │
│ gene by assembly PCR        │
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              │
              ▼
┌─────────────────────────────┐
│ Clone into pCR ®2.1         │
│ ®TOPO cloning vector        │
└─────────────────────────────┘
              │
              ▼
     ┌──────────────────┐
     │ DNA sequencing   │
     └──────────────────┘
              │
              ▼
┌─────────────────────────────┐
│ Clone into shuttle plasmids │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│ Transformation into BCG     │
└─────────────────────────────┘
              │
              ▼
┌──────────────────────────────────────┐
│ SDS PAGE and Western Blot to detecy   │
│ expression of viral protein in rBCG   │
└──────────────────────────────────────┘
              │
              ▼
     ┌──────────────────────┐
     │ Studies in mice model│
     └──────────────────────┘
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Immunogenicity ──Serum──▶ Analyses of IgG level in serum by ELISA

Splenocyte

- Lymphocyte proliferation assay (LTT)
- Analyses of intracellular cytokines by flow cytometry
- Analyses of extracellular cytokines by ELISA

~~Fig.~~Fig. 1.~~4~~5: Flow chart for cloning, expression and animal studies.

CHAPTER 2

MATERIALS AND METHODS

This ~~section~~section describes the materials and general methods used in this work. Specific methods are described in the respective chapters.

~~2.1~~2.0 Materials

~~2.1.1~~2.0.1   Mice

Six to 8 week-old BALB/c (H-2^d) female mice were obtained and housed in a pathogen-free environment at the animal house of the Universiti Sains Malaysia (USM), Health Campus, Kelantan. All animal care and experimental procedures were performed according to guidelines set by the Malaysian Association for Accreditation of Laboratory Animal Care and this work was approved by ethical committee, USM. They were given water and standard food pellet *ad libitum.*

~~2.1.2~~2.0.2   Bacterial strains, culture media and growth conditions

Different *E.coli* strains were used for a variety of purposes during this study; these strains are listed in Table 2.1. Liquid and plate cultures as well as glycerol stocks