Appl Microbiol Biotechnol (1991) 36:1-4
017575989100231F

Applied Microbiology and Biotechnology

Mini-review

On the safety of *Bacillus subtilis* and *B. amyloliquefaciens*: a review

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Received 28 May 1991/Accepted 6 June 1991

Introduction

For many years the fermentation industry has used microorganisms to produce antibiotics, amino acids, enzymes and other useful compounds. These microorganisms, which have been isolated from the environment and then mutated to increase yields of the desired product, have proved safe to handle. With the advent of gene technology, it is now possible to transfer genetic properties from one organism to another. It is widely accepted that as long as the recipient microorganism (the host) is harmless and the products of the genes to be transferred are innocuous, the genetically engineered microorganism (the recombinant) is as safe as the host.

An overwhelming majority of recombinant microorganisms to be used by industry are expected to be based on harmless hosts (OECD 1986). Many of these have been proven safe over many years of experience in industrial settings. Furthermore, extensive information on the incapacity to cause disease, i.e. non-pathogenic and non-toxicogenic potential, of some of these organisms can be found in the literature.

We believe that a review of the literature and present experience with some of these host organisms will be useful for assessment of the safety of many recombinant organisms. In particular it may help to classify some of these as GILSP (Good Industrial Large Scale Practice) host organisms as defined by the OECD (1986), thus facilitating the use of recombinant strains by established production procedures. Furthermore, safety reviews on selected host microorganisms may ease the approval process of products produced by recombinant strains derived from these hosts. Thus it is the opinion of qualified experts that the use of genetic engineering per se does not warrant any additional safety assessment. On the contrary, use of a safe and well-known host organism may sometimes render superfluous some of the extensive animal testing of a new product. For a more extensive discussion of the safety and regulatory aspects of the use of recombinant organisms see, for example, AMFEP (1990), Diderichsen et al. (1990), IFBC (1990), National Academy of Sciences (1987), and Pariza and Foster (1983).

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Taxonomy and ecology

Bacillus subtilis is a Gram-positive, spore-forming bacterium. It is commonly found in soil and on plant material and grows aerobically at intermediate temperatures and pH. As with many other bacilli, *B. subtilis* secretes substantial amounts of protein, especially hydrolytic enzymes such as amylases and proteases. *B. subtilis* is often referred to as a non-pathogenic bacterium and it is even consumed by humans in large quantities in the Japanese food natto (Djien and Hesseltine 1979).

The genetics of *B. subtilis* strain 168 (Burkholder and Giles 1947) has been extensively studied, making it the best characterized Gram-positive bacterium. *B. amyloliquefaciens* was first isolated in 1943 and the suggested distinction from *B. subtilis* (Welker and Campbell 1967) is now well documented. *B. amyloliquefaciens* has been given separate species status and its name has been included on the approved lists of bacterial names (Priest et al. 1987).

Industrial uses

Bacilli are widely used by the fermentation industry. Well-known examples are *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. alkalophilus*, *B. lentus* and *B. thuringiensis*. For a recent review on the biotechnology of bacilli see Priest (1990).

Safety aspects

In general, *B. subtilis* is considered an opportunistic microorganism with no pathogenic potential to humans. However, *B. subtilis* is virtually ubiquitous and it is therefore inevitable that it sometimes may be found in association with other microorganisms in infected humans, but only patients treated with immunosuppressive drugs appear to be susceptible to infection with this otherwise harmless microorganism (Doyle et al. 1985). We have attempted to collect all pertinent references reporting such cases and to analyse whether *B. subtilis* can cause human disease. We mainly refer to cases described after 1970 as confusion between *B. cereus* and *B. subtilis* existed in diagnostic laboratories before that time (Gordon 1973).

B. subtilis, as well as other *Bacillus* species, is an important occupant of most environments. A survey by Finch et al. (1978) of the bacterial flora at different sites in 21 homes showed that *Bacillus* species were present at all of 17 sites in the kitchen and all of 16 sites in the bathroom. Together with *Micrococceae*, *Bacillus* spp. were the most frequent organisms isolated. This is probably due to the common occurrence in soil of bacilli combined with their ability to produce spores.

Infections

Several authors have noted an increased frequency of registration of infections with *Bacillus* species (Logan 1988; Kramer and Gilbert 1989). As stated by Logan (1988), this might be associated with improved bacteriological techniques and the increasing number of severely debilitated patients, for example those who are immunologically compromised.

The literature describing human infections with *B. subtilis* has been collected from database searches and from our collection of references on *Bacillus* pathogenicity. The search resulted in less than ten relevant articles describing approximately 50 cases of putative *B. subtilis* infections. Note that this figure is extremely low considering the total number of reports on bacterial infections. Almost all cases were related to drug abuse or occurred in severely debilitated patients.

Drug abuse. In drug abusers Tuazon et al. (1979) described four incidents of endocarditis (i.e. inflammations of the heart). *B. cereus* was isolated in all cases. Reller (1973) describes one case of endocarditis caused by *B. subtilis* in a drug abuser.

Infections of drug abusers by bacilli are related to the fact that narcotics are often contaminated by bacilli. Thus, the presence of *Bacillus* species in narcotics for intravenous administration has been examined. Shamsuddin et al. (1982) investigated 49 heroin samples and found 20 to be contaminated. Of these 13 were contaminated by *Bacillus* spp. In a separate study, 47% of the injection utensils and 32% of heroin samples were found to be contaminated by *Bacillus* species (Weller and Nicholson 1979).

Debilitated patients. Ihde and Armstrong (1973) reported on 12 cases of *Bacillus* spp. infections during a 5-year period from 1966 to 1971. The patients suffered from malignant cancer diseases. Ten of the cases were described as *B. subtilis* infections, but as the data were collected before *B. cereus* and *B. subtilis* were clearly distinguished from each other, the diagnosis may well be erroneous. Pennington et al. (1976) described two cases of *B. subtilis* infection in two patients suffering from blood cancer. *B. subtilis* was isolated from lung and brain tissue.

In a retrospective examination of cases of *Bacillus* spp. isolated from blood samples at a hospital with a large proportion of immunosuppressed patients, Cotton et al. (1987) analysed 17 cases from a 9.5-year period. Fourteen of the patients had chronic venous catheters and *B. subtilis* was not found in any of the blood samples.

Kiss et al. (1988) reported on 21 *B. subtilis* bacteremias in patients all suffering from debilitating diseases. The treatment of the primary disease in all patients included insertion of intravenous catheters, lumbar puncture or other interventions, which may have introduced the organism to sensitive tissue.

Richard et al. (1988) described 11 cases of *Bacillus* bacteremias of which *B. subtilis* was isolated in eight patients. Four of these suffered from cancer diseases and four others had head trauma, stroke or had undergone surgery. A routine of using *B. subtilis* culture as a non-specific support for a stable gastrointestinal flora was suspected of being responsible for the infections.

Local infection. Infections of the eye by *B. cereus* has caused irreversible loss of sight (Shamsuddin et al. 1982). According to literature after 1970, however, *B. subtilis* seems not to be the agent of infections of the internal eye. Donzis et al. (1988) reported on a case of *B. subtilis* eye infection related to contamination of contact lenses. Jonas et al. (1981) reported on one case of infection in the shin-bone of a 1-year-old child caused by a splinter in the growth plate of the bone.

Food poisoning. B. cereus is well-established as a cause of food poisoning accounting for 1–23% of the reported foodborne illnesses in humans (Kramer and Gilbert 1989). *B. subtilis* has been isolated in some cases of food poisoning, but the number of episodes is low. Thus, Kramer and Gilbert (1989) reported on only 49 episodes in the UK in the period 1975–1986. Exact and reliable figures are difficult to obtain, since *B. cereus* sometimes may have been classified as *B. subtilis*. As a consequence, there are very few examples of *B. subtilis* as the confirmed cause of food poisoning.

B. amyloliquefaciens. B. amyloliquefaciens has not appeared in any of the cited papers dealing with *Bacillus* sp. as infectious organisms. A search in databases for references on *B. amyloliquefaciens* infections or intoxications revealed no such cases, probably because Gordon et al. (1973) considered *B. subtilis* and *B. amyloliquefaciens* synonymous.

Recombinant strains

Since the discovery of plasmids that are able to replicate in *B. subtilis* (Ehrlich 1977), *B. subtilis* 168 has been used as a host for cloning DNA of both prokaryotic and eukaryotic origin. Considering *B. subtilis* harmless, the National Institute of Health (US) has exempted sporulation-deficient strains from its Guidelines for Research Involving Recombinant Molecules (May 7, 1986). On August 24, 1987, the NIH modified the Guidelines (Appendix C-IV) such that the physical containment of large-scale fermentation experiments involving sporulation-deficient recombinant *B. subtilis* does not need to be greater than for the unmodified host.

Permission to produce enzymes from recombinant *B. subtilis* strains have been given in the US, Japan, and Denmark and the Danish Ministry of Health has issued an environmental certification stating that a recombinant *B. subtilis* production strain comply with the OECD recommendations on Good Industrial Large Scale Practice organisms (OECD 1986).

Bielecki et al. (1990), described cloning of the structural gene for *Listeria monocytogenes* haemolysin, *hlyA*, into an asporogenic *B. subtilis* strain. The recombinant, in contrast to the host strain, was able to grow in vitro in the cytoplasm of macrophage-like cells after being internalized. However, the recombinant was absolutely avirulent after intervenous injection in mice and thus did not display any pathogenic properties in vivo. This is in accordance with the general belief that pathogenicity is a multifactorial property.

The FDA (Food and Drug Administration of the US) may grant products the status of being "Generally Recognized As Safe" (GRAS). Evidence, which in FDA's opinion may lead to this conclusion is published for public comment as a GRAS petition, which eventually may lead to clearance as GRAS.

In a GRAS petition by CPC International (1986), the company reviewed the pathogenicity and toxicogenicity of *B. subtilis*. A search covering the period 1907–1983 failed to disclose a single report demonstrating that *B. subtilis* can be the etiological agent of diseases in man or animals. In the GRAS petition it is noted that although *B. subtilis* strains have sometimes been reported to be implicated in food poisoning, the reports are speculative and in no cases were confirmatory toxicological studies conducted. In the same GRAS petition, specific toxicological studies showed that an α-amylase from *B. stearothermophilus* produced by a recombinant *B. subtilis* is safe for use in food. In another GRAS petition Enzyme Bio-Systems (1988) demonstrated the safety of a *B. megaterium* amylase produced by a recombinant *B. subtilis*. Finally, a GRAS petition from Novo Laboratories (1990) included safety data on a maltogenic amylase produced by a recombinant *B. subtilis*. Andersen et al. 1987 published a safety study on the toxicological and mutagenic potential of the same enzyme.

Conclusion

No case demonstrating invasive properties of *Bacillus subtilis* or *B. amyloliquefaciens* has been described but in a few cases, *B. subtilis* has been found associated with drug abusers or severely debilitated patients. Thus there is no evidence of any pathogenic potential of *B. subtilis* to humans in general. *B. subtilis* has been associated with some cases of food poisoning which in part may be due to misclassification of *B. cereus*. Thus there are very few examples of *B. subtilis* strains as confirmed causes of food poisoning. We conclude that *B. subtilis* is a safe host for the production of harmless products.

Acknowledgement. We are grateful to Dr. Fergus Priest, Edinburgh, for critical comments on the manuscript.

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