Highlight on Engineering *Mycobacterium smegmatis* for testosterone production

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**Background**

The development of male attributes such as external genitalia and secondary sexual characteristics is due to a hormone called testosterone produced in the testicles. This hormone is also responsible for the development and maintenance of muscle mass (Griggs et al., 1989), bone density, red blood cell counts (Bachman et al., 2014), supports sexual and reproductive function and contribute to a man’s sense of anger and vitality (Batri-nos, 2012).

While the underlying mechanism is not known, the production of testosterone in males gradually declines with age, beginning at around 30 (Morley and Perry, 2000). The decline is associated with a near-total lack of interest in sex, erectile dysfunction (Kratzik et al., 2005), diabetes (Barrett-Connor, 1992), Alzheimer’s disease (Moffat et al., 2004), cardiac failure (Kontoleon et al., 2003), hypercholesterolaemia (Haffner et al., 1993), osteoporosis (Campion and Maricic, 2003), frailty, obesity (Svatberg et al., 2004), hypertension (Phillips et al., 1993) and ischaemic heart disease (Barrett-Connor and Khaw, 1988). The idea of supplementing declining levels of testosterone to treat diseases was realized by the scientific community as early in the mid-1930s (Hamilton, 1937). In recent years, the use of testosterone therapy has become more widespread, but the only available synthetic form of testosterone is still expensive. Hence, a very little fraction of those affected are able to afford this therapy.

Microbes are ubiquitous and humans have exploited their biological processes, especially through genetic manipulation for the production of hormones and antibiotics. In 1978, genes encoding human insulin was cloned and expressed in *E. coli* followed by cloning of growth hormone in 1979, which replaced the unhuman forms of both these hormones. However, until now, testosterone (TS) has been chemically produced from androst-4ene-3,17-dione (AD) (Ercoli and Ruggieri, 1953). Not only being expensive, the synthetic form has been reported to induce several side-effects such as allergy, nausea or vomiting, impotency, painful or difficult urination, high levels of calcium in the blood, mild truncal acne, weight gain (Matsumoto, 1990) and coronary heart disease (Tripathy et al., 1998). In mammals, 17-ketosteroid reductase (17β-HSD) enzyme catalyses the synthesis of TS from AD. There was a hunt for microbial sources — both bacterial and fungal enzymes (Donova et al., 2005) that could convert AD to TS in order to reduce the production cost and benefit patients allergic to the synthetic derivative of TS. The ability of microorganisms to reduce 17-keto- to 17β-hydroxysteroids was first reported in *Saccharomyces cerevisiae*, during the transformation of androst-4ene-3,17-dione to testosterone (Charney and Herzog, 1967). Subsequently, the ability to carry out 17β-reduction of AD was reported for a variety of microorganisms of different taxonomy, including *Mycobacterium, Pediococcus, Brevibacterium, Bacillus, Arthrobacter, Lactobacillus* and *Nocardia* (Wix et al., 1968; Uwajima et al., 1973; Mahato and Mukherjee, 1984; Dutta et al., 1992; Kumar et al., 2001). But, very few microbial 17β-OH SDHs were isolated and characterized. The most investigated enzyme is 3(17)β-hydroxysteroid dehydrogenase (3(17) β-OH SDH) isolated from *Comamonas testosteroni* (earlier classified as *Pseudomonas testosteroni*) (Groman and Engel, 1977). Attempts were made to introduce the 17β-hydroxysteroid gene from *Comamonas testosteroni* in *E. coli* (known model system for biotechnological processes) (Pliésiat et al., 1991). However, substantially low uptake of AD across the cellular membrane by *E. coli* limited the production process instigating researchers to look for novel biological models for the industrial production of...
testosterone. Considering the cost and the side-effects, attempts have been made to produce testosterone in vitro, from AD using recombinant murine 17β-HSD type V and glucose dehydrogenase as a cofactor (Fogal et al., 2013). However, this approach was not employed for industrial purposes due to the high cost of the production process.

A recent article by Fernández-Cabezón et al. (2016) proposes a biological model for industrial production of testosterone using Mycobacterium smegmatis. This model overcome the first major bottleneck, that is uptake of AD by a bacterium. M. smegmatis can efficiently transport AD across its cellular membrane and does not degrade AD. Therefore, it was engineered for the biotransformation of AD to TS. For this, two genes — one from the bacterium Comamonas testosteroni and the second from the fungus Cochliobolus lunatus encoding microbial 17β-hydroxysteroid:NADP 17-oxidoreductase — were selected and introduced in the host cells. The host strains were M. smegmatis (wild-type) and a genetically engineered androst-4-ene-3,17-dione (AD)-producing mutant. First, Fernández-Cabezón and co-workers cloned both these genes in pMV261, an E. coli/M. smegmatis shuttle vector under the control of a constitutive promoter. This was then followed by cloning of these two genes in two different plasmids (pHSDCT and pHSDC) that were transformed in wild-type and mutant M. smegmatis strain. The recombinant strains were able to produce TS from sterols or AD with high yield when compared with the production by mycobacterial strains obtained by conventional mutation procedures (Liu et al., 1994; Liu and Lo, 1997).

This process for the production of testosterone (TS) has been developed to compete with current chemical synthesis procedures. The major obstruction in bringing this biotechnological process from laboratory to industrial scale production will be optimizing the production of TS from sterols in a single biotransformation step. The reversibility of 17β-HSD enzymes and cell metabolic state are two most important determinants for improvement of this process. Attempts are being carried to design mutants of 17β-HSD having improved substrate specificity and coenzyme requirements. The host cell’s metabolic state should also be modified using carbon source supplements, determining adequate pH or varying the mode of substrate addition that will help in efficient reduction of AD to TS (Fig. 1). This will take time and is a very long process, but when successfully accomplished will replace the current procedure of chemical synthesis. This work also opens up the possibilities of producing TS analogues with better ability by genetic manipulations in the genes responsible for TS formation. The stable analogues could have a better shelf life and improved efficiency. Even though the work provides a proof of concept to start with, this is still a major achievement. This work has opened up possibilities of this model bacterium being used for the production of important pharmaceutical steroids using metabolic engineering approaches.

Conflict of interest

None declared.

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Fig. 1. Hydrogenation reaction of Androst-4ene-3,17-dione to testosterone by 17β-hydroxysteroid dehydrogenase.
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